

# Cardiovascular Morbidity in Juvenile-Onset Systemic Lupus Erythematosus

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I, Catherine Quinlan, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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# Abstract

Cardiovascular disease (CVD) is a leading cause of mortality in adults with systemic lupus erythematosus (SLE). As children with conditions such as chronic kidney disease have been shown to have a risk of CVD similar to their adult counterparts, clinicians have become concerned that paediatric patients with SLE are at increased risk of CVD.

The aim of this project was to examine the vascular phenotype of a British paediatric population with SLE and gain mechanistic insights into this process. Structural changes in vessels were measured using carotid intima media thickness (cIMT) and function was assessed using pulse wave velocity (PWV). These findings were compared with clinical information, traditional cardiovascular risk factors, disease activity, medications and adipokine activity.

45 children were recruited to the study and compared to historical controls previously studied in our centre. Children with SLE had higher cIMT than controls ( $0.45 \pm 0.37\text{mm}$ ,  $p < 0.0001$ ) but no difference in PWV ( $5.27 \pm 5.34\text{m/s}$ ,  $p = 0.77$ ). The increase in cIMT is most marked in patients with hypertension, those on higher doses of prednisolone and those of Afro-Caribbean descent. No significant association was found between increased cIMT and biopsy-proven nephritis, disease activity, age, family history of CVD or physical activity score.

Patients with JSLE had increased serum leptin levels ( $15.5 \pm 7.56\text{ng/ml}$ ,  $p = 0.024$ ). There were slightly higher adiponectin levels in patients than controls ( $14.2 \pm 12.4\text{ug/L}$ ,  $p = 0.49$ ). Patients with proteinuria had higher leptin ( $35.5 \pm 18.8\text{ng/ml}$ ) and adiponectin ( $21.9 \pm 10.5\text{ug/ml}$ ,  $p = 0.03$ ) levels, and cIMT increased with leptin and adiponectin levels.

This cohort of children with JSLE show structural changes in their vessels indicative of early CVD but with adaptive changes resulting in normal functional scans. In contrast to adult data this group displays an early increase in serum adiponectin suggesting a possible early protective mechanism, which may be overwhelmed in later disease.

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# Abbreviations

1,25 (OH) <sub>2</sub> D	1,25 dihydroxyvitamin D3
25 (OH)D	25 hydroxyvitamin D
Ab	Antibody
ACE-inhibitor	Angiotensin Converting Enzyme Inhibitor
ACR	American College of Rheumatology
ADDIT	Adolescent type 1 diabetes cardio-renal Intervention trial
ALSPAC	Avon longitudinal study of parents and children
Ang2	Angiopoietin-2
Anti-C1q	Antibodies to C1q complement factor
Anti-dsDNA	Antibodies to double stranded DNA
ANZDATA	Australia and New Zealand Dialysis and Transplant Registry
aPL	Antiphospholipid

ApoA1	Apolipoprotein A-1
APPLE	Atherosclerosis Prevention in Paediatric Lupus Erythematosus
AS	Atherosclerosis
BILAG	British Isles Lupus Assessment Group
BMI	Body mass index
cIMT	Carotid intima media thickness
CKD-MBD	Chronic kidney disease mineral bone disease
CRP	C-reactive protein
CT	Computed tomography
CV	Cardiovascular
CVD	Cardiovascular disease
DICOM	Digital Imaging and Communications in Medicine
EC	Endothelial cell
ECG	Electrocardiogram

EPC	Endothelial progenitor cell
FGF-23	Fibroblast growth factor 23
FMD	Flow mediated dilatation
GM-CSF	Granulocyte macrophage colony-stimulating factor
GOSH	Great Ormond Street Hospital for Children NHS Foundation Trust
HDL	High density lipoprotein
hsCRP	High Sensitivity C-reactive Protein
HTN	Hypertension
ICAM-1	Intercellular cell adhesion molecule-a
ICH	Institute of Child Health
IFN	Interferon
IFN- $\alpha$	Interferon- $\alpha$
IFN- $\gamma$	Interferon- $\gamma$
IL-1, -6, -10	Interleukin-1, -6, -10



IPEX Syndrome	X-linked, immunodystrophy, polyendocrinopathy and enteropathy
IRA B-cells	Innate response activator B-cells
JSLE	Juvenile-onset systemic lupus erythematosus
LDL	Low density lipoprotein
LDL	Low density lipoprotein
LN	Lupus Nephritis
LVH	Left Ventricular Hypertrophy
MCP-1	Monocyte chemotactic protein-1
MDCT	Multi-detector computed tomography
METS	Metabolic equivalent of task
MMF	Mycophenolate mofetil
mTOR	Mammalian target of rapamycin
NF- $\kappa$ B	Nuclear factor- $\kappa$ B
NO	Nitric oxide

PDGFR $\beta$	Platelet Derived Growth Factor Receptor- $\beta$
piHDL	Pro-inflammatory high density lipoprotein
PWV	Pulse wave velocity
RBP4	Retinol binding protein-4
RCH	Royal Children's Hospital
SBP	Systolic Blood Pressure
SD	Standard deviation
SLE	Systemic lupus erythematosus
SLEDAI	Systemic lupus erythematosus disease activity index
SLICC	Systemic Lupus International Collaborative Clinics
SLICC/ACR	Systemic Lupus International Collaborative Clinics/American College of Rheumatology
SMC	Smooth muscle cell
SPECT	Single photon emission computed tomography
TG	Triglyceride

TGF- $\beta$ 1	Transforming Growth Factor- $\beta$ 1
Th1	Type 1 T-helper cells
Th2	Type 2 T-helper cells
TLR	Toll like receptor
TLR4	Toll-like receptor-4
TNF	Tumour necrosis factor
Treg	T-Regulatory cells
UaUc	Urine albumin to creatinine ratio
UCL	University College London
UKJSLE cohort	United Kingdom Juvenile-onset Systemic Lupus Erythematosus cohort
VCAM-1	Vascular cell adhesion protein-1
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell
vWf	Von willebrand factor

# Introduction

Cardiovascular disease (CVD) was first identified as a major cause of mortality in adults with systemic lupus erythematosus (SLE) in the 1970s but its aetiology remains poorly understood. It is unclear whether SLE (or the treatment of SLE) acts as an independent risk factor or whether the increased risk of CVD in adults is due to a clustering of traditional cardiovascular (CV) risk factors.

SLE is a lifelong autoimmune disease, affecting multiple organ systems and leading to a relapsing and remitting chronic inflammatory state. Diagnosis is usually made from the clinical presentation, classical system involvement and abnormal autoantibodies. For research purposes patients are classified according to the American College of Rheumatology Criteria(1,2), see table 1. Of these criteria at least 4 are required to be present, over the course of the disease, to classify the patient as having SLE.

Over the past two decades it has become clear that CVD is in itself an inflammatory process and that individuals with chronic inflammatory disease, such as SLE or rheumatoid arthritis, are at increased risk. As children with diseases such as chronic kidney disease (CKD) have been shown to have a risk of CVD similar to their adult counterparts, clinicians have worried that paediatric patients with SLE are at increased risk of CVD.

While it remains unclear in the adult population whether increased CV risk is a general feature of SLE or whether it only affects select subgroups of patients there is certainly an increased risk of CVD. Indeed, even after controlling for the traditional Framingham risk factors the excess risk of CVD in SLE is still 7.5-fold higher than the general population.(3)

## Aims

The aim of this project is to examine CV risk in children with juvenile-onset systemic lupus erythematosus (JSLE), to determine whether there is an increased risk of CVD and to analyse its epidemiology in an effort to understand its pathogenesis. Since children are highly unlikely to experience a hard CV endpoint, such as a myocardial infarction or a cerebro-vascular accident, validated measures of sub-clinical CVD will be used as surrogate markers for CV risk.

## Hypotheses

1. Children with JSLE have a low prevalence of the classical modifiable risk factors for CVD, obesity, inactivity and smoking, and thus represent a “clean” population in which to study the effect of SLE and the treatment of SLE on the vascular phenotype.
2. JSLE is associated with an increased risk of CVD and that this is associated with an abnormal vascular phenotype. This will be ascertained by measuring well-validated measures of sub-clinical CVD to establish the vascular phenotype of children with JSLE.
3. The increased risk of CVD in JSLE is further exacerbated by the presence of hypertension, renal impairment, the use of corticosteroids and the duration of disease.
4. The increased risk of CVD in JSLE is related to increased disease activity leading to abnormalities in serum lipids and adipokine activity followed by the development of an abnormal vascular phenotype.

### Study value

This study is novel in aiming to establish the vascular phenotype in the British population with JSLE and attempting to gain mechanistic insights into this process. It builds on adult research and offers new insights since the JSLE cohort are too young to have a significant burden of CV risk secondary to modifiable risk factors for CVD.

# Background



## Introduction

In this chapter I will review the literature regarding cardiovascular disease and SLE, presenting the evidence for an increased risk of CVD in adult onset SLE and the potential pathogenic factors. I will outline the current understanding of the pathogenesis of both atherosclerosis and SLE, in order to describe the connection between these two chronic inflammatory diseases. I will start with an overview of SLE to discuss its epidemiology, outlining the clinical features, serology, treatment and outcomes of children and adults with SLE.

## Epidemiology of SLE

SLE is a severe, episodic, chronic, autoimmune disease, whose hallmark is the formation of autoantibodies against nuclear antigens. It has a variable presentation and clinical course with multi-system inflammation affecting the skin, joints, kidneys heart, lungs and nervous system. The pathogenesis of SLE is multifactorial disease with hormonal, genetic and environmental influences. Its management is multidisciplinary involving medical and nursing staff from nephrology, rheumatology and dermatology along with physiotherapy, occupational therapy and psychological patient support.

Patient survival in SLE has improved considerably over the last few decades, where once there was high early mortality, 5-year patient survival has improved from 64-87% in the 1980's to greater than 95% today. Improved patient survival has led to greater disease prevalence. In a 2007 review D'Cruz et al outlined disease prevalence by country comparing the US population with a prevalence of 52.2 per 100,000, the UK with 26.2 per 100, 000 and Japan with 28.4 per 100,000. (4)

Differences in prevalence and outcomes between ethnicities point to a genetic component in the progression of this disease. Certainly there are ethnic differences in disease progression. In the UK, where medical care is free and thus socio-economic status, ethnicity and outcomes are easier

to delineate, Afro-Caribbean patients with renal disease were more likely to go into renal failure than Caucasian patients. (5)

**Table 1. 1997 Update of the 1982 American College of Rheumatology Revised Criteria for Classification of Systemic Lupus Erythematosus**

Criteria	Notes
<b>Malar Rash</b>	
<b>Discoid Rash</b>	
<b>Photosensitivity</b>	
<b>Oral Ulcers</b>	
<b>Nonerosive Arthritis</b>	Involving $\geq 2$ peripheral joints
<b>Pleuritis or Pericarditis</b>	
<b>Renal Disorder</b>	Persistent proteinuria $>0.5\text{g}/24\text{hrs}$ or 3+ Cellular Casts
<b>Neurological Disorder</b>	Seizures Psychosis
<b>Haematologic Disorder</b>	Haemolytic anaemia Leukopenia $\geq 2$ occasions Lymphopenia $\geq 2$ occasions Thrombocytopenia
<b>Immunologic Disorder</b>	Anti-DNA Ab Anti-Sm Ab Positive Antiphospholipid Ab
<b>Positive Antinuclear Antibody</b>	

In adults, SLE chiefly affects women, with a female to male ratio of 8:1, although JSLE affects more boys, with a ratio of 5.6:1, it remains a largely female disease. (6) This difference is thought to be due to interactions between the immune system and female hormones. Although some studies, including a prospective cohort study of 1000 European patients followed from 1991, have shown that men with lupus have a higher rate of serositis and a lower rate of arthritis, others, including a single centre, observational study of 484 patients over 30 years, have shown very little difference when data is analysed by gender. (7-10)

Drug treatments are outlined in greater detail later in this chapter but include steroids, non-steroidal anti-inflammatory drugs, hydroxychloroquine, azathioprine, methotrexate, cyclosporine, cyclophosphamide, mycophenolate mofetil, tacrolimus and intravenous immunoglobulin (IVIg) along with newer biological agents including rituximab, belimumab and eculizumab.

Although the majority of individuals present in adulthood, with a mean age of diagnosis of 34 to 51 years (11) in one registry, (12) 15-20% of patients present to paediatric services. (13,14) The incidence of JSLE is 0.3-0.9 per 100,000 children per year with a higher incidence among Asian and Afro-Caribbean populations. (15) Despite the improvements in outcomes for adults-onset SLE, patients with JSLE continue to have a lower life expectancy than the general population and in comparison to adults with SLE (16-19)

Paediatric data has consistently shown that patients with JSLE have a higher incidence of arthritis, LN, neurological manifestations and haematological involvement. (6,19,20) Half of patients with JSLE will present in adolescence and the median time from symptom onset to diagnosis is 3.2 months, reflecting the difficulty of diagnosing a rare, multisystem disorder with frequently nonspecific symptoms at presentation. After 4 – 5 years of follow up, 80% of patients have

experienced renal involvement, 90% have experience haematological involvement, 80% have experienced musculoskeletal disease and 26% have experienced neurological disease. (6)

With regards to serology one large UK cohort has compared 124 patients with JSLE and 484 adults with SLE managed in the same unit, (19) observed a trend towards lower serum complement in the JSLE group. However this did not reach statistical significance and overall there was no significant serological difference between the groups.

Children and adolescents with SLE are not just little adults, they have more severe disease at presentation, a greater prevalence among boys, a more aggressive disease course and a marked increase in mortality when compared with their adult counterparts. Patients with JSLE are more likely to present with fatigue, lupus nephritis (LN), haematological involvement (particularly haemolytic anaemia) and neurological disease (especially seizures). They will have a greater lifetime burden of disease, are more likely to require long-term immunosuppression and must cope with the added challenges of adolescence and transition to adult services. (6,15,17-19,21,22)

### **Adult-onset SLE is associated with increased CVD**

Adults with SLE were first identified as being at increased risk of CVD over 35 years ago by Urowitz et al (23) who identified a bimodal pattern of mortality in a cohort of adults with SLE. Half of the deaths in this cohort occurred within a year of diagnosis due to active SLE, and the remainder at a mean of 8.6 years with a recent myocardial infarction. Early deaths were largely due to infectious complications.

The management and treatment of SLE has improved dramatically in the intervening decades with the advent of tailored, targeted therapies such as anti-B and T cell treatments and the improvements in multi-disciplinary team management. This has resulted in a lower mortality

rate and a higher quality of life for patients living with SLE. However, similar to many chronic conditions, as patients have lived longer newer long term risks have become evident.

In the years since Urowitz published his landmark paper the increased risk of CV events among adults with SLE has been confirmed by a number of prospective studies which have demonstrated an increased relative risk of CV events in individuals with SLE when compared to their unaffected peers. However much of the improvement in mortality secondary to CVD are due to improvements in the overall management of CVD and not due to advances in the understanding of the mechanisms underpinning CVD in SLE.

Bernatsky et al (24) examined a very large international cohort of 9547 patients with SLE over a mean of 8.1 years. During the observation time 1255 deaths occurred, with circulatory disease identified as the cause of death in 313 cases. This gave a standardised mortality ratio of 2.4 and a standardised mortality ratio of 1.7 for death due to circulatory disease. Most notably patients aged less than 25-years had the highest rates of death due to SLE with 5.3 per 1000 person years versus 2.5 for the group overall. Cause of death was ascertained by linkage to the National Death Index in the US and with regional vital statistics registries for non-US patients and this is open to misinterpretation if a post-mortem has not been performed, nonetheless it underlines again the increased mortality in SLE.

Similarly Ward (25) examined hospitalisation for acute myocardial infarction, cerebrovascular accident or congestive heart failure for women with SLE in California from 1991 to 1994, showing that women aged 18-44 with SLE were 2.27 times more likely to be hospitalised because of a myocardial infarction and 2.05 times more likely to be hospitalised because of a cerebrovascular accident. Each woman was compared with 5 women who did not have SLE. However since the data

set limited itself to hospitalised patients and so could not estimate population odds ratios and restricting analysis to only cardiovascular diagnoses that are objectively verifiable could underestimate disease prevalence. More recently, Urowitz et al (26) described a large, multinational cohort of patients with SLE recruited prospectively and followed for 8 years. These 1,249 patients have had 97 vascular events including 13 myocardial infarctions. Fifty of the events were attributed by the patient's physician to active SLE, and 31 to atherosclerosis. Examining just the events that the treating clinician attributes to atherosclerosis dilutes the findings somewhat, but certainly shows an increased rate of CVD in SLE. In contrast to Manzi they showed a mean time from diagnosis to first cardiovascular event of just 2 years but a much broader definition of a cardiovascular event was used (myocardial infarction, angina, congestive heart failure, peripheral vascular disease, transient ischaemic attack, stroke or pacemaker insertion) and only found age and male gender to be associated with atherosclerotic events.

Before examining the pathogenesis of CVD in SLE I will firstly put forward some of the evidence to support the claim that there is an increased risk of CVD in SLE.

### **Epidemiological evidence for increased prevalence of CV events in adult-onset SLE**

Several cohort studies have compared healthy individuals and those who have developed SLE and have demonstrated an increased risk of CVD in adults with SLE when compared to their peers.

The Nurses' Health Study(12) represents an excellent opportunity to examine the risk of CVD in a prospectively followed cohort of healthy women, a proportion of whom were subsequently diagnosed with SLE. Almost 120,000 healthy women have been followed for 28 years since enrolment in 1976. All were free of a diagnosis of SLE or CVD at enrolment. In this study a CV event was defined as a myocardial

infarction, stroke, coronary artery bypass grafting or angioplasty. Myocardial infarction was defined as symptoms plus either diagnostic electrocardiographic (ECG) changes or elevated cardiac enzymes. Stroke was defined as neurological deficit with rapid onset that persisted for >24 hours or until death.

In 28 years of follow-up (2.9 million person-years) 8169 CV events occurred and 148 women were diagnosed with SLE. Mean age of diagnosis of SLE was 52.6 years. Amongst the SLE group there was a greater prevalence of diabetes, hypertension (HTN) and a positive family history of CVD before the age of sixty. The age adjusted relative risk of any CV event was 2.75 for participants with SLE and a 1-year increase of SLE duration was associated with a relative risk of 1.08 for CVD. After adjusting for age, ethnicity, CV risk factors and medication use the relative risk of a CV event in women with SLE compared to those without was 2.26. The relative risk for coronary heart disease was 2.25 and for cerebrovascular accident was 2.29. Thus demonstrating a clearly increased risk of CVD in women with SLE.

Similarly the following SLE register studies have followed adults diagnosed with SLE longitudinally and have shown an increased prevalence of CVD when compared with the general population. Different studies have used different definitions of CV events making it challenging to compare results. However, they have consistently shown an increased rate of CV events in the SLE population with an even greater relative risk in the younger SLE population, remembering that this is affected by the low baseline risk of CVD in the younger, healthy population.

A registry study (27) of 4737 Swedish patients diagnosed with SLE between 1964 and 1994 were followed by linkage to the Cause of Death Register. This showed a 3-fold increased risk of death with CVD the main cause of premature death when compared with the Swedish population.

Even more concerning was the finding that patients aged 20 to 39 years showed a 16-fold risk of death from coronary heart disease. Mortality was analysed separately in three 10-year periods (1964-1975, 1975-1984 and 1985-1995) and this showed a decrease in all-cause mortality since 1975. However, the reason for this decrease was due to a decrease in death due to active SLE with no change in death secondary to CVD.

Gustafsson et al (28) followed 182 patients with SLE, free of CV events at first assessment, for a mean of 8.3 years. At inclusion the cohort had a mean age of 43.9 years and 24 (13%) had a first CV event during follow up. CV events were defined as myocardial infarction, cerebral infarction, transient ischemic attack, intermittent claudication, peripheral arterial thrombosis, peripheral arterial embolus or death due to myocardial infarction, heart failure, sudden death, cerebral infarction or generalized atherosclerosis. This is a more generous definition of a CV event than that used in the Nurses' Health Study (12). Patients who developed a CV event were more likely to be older, to have smoked, to have higher blood pressure and to have higher cholesterol.

Likewise in a 12-year follow up of 156 patients with SLE, by Becker Merok et al, (29) 41 (26%) had a vascular event. Vascular events were defined as myocardial infarction, angina pectoris, intermittent claudication, cerebrovascular accident, deep venous thrombosis, pulmonary embolism, intestinal infarction not related to vasculitis, avascular necrosis of the bone, acute arterial ischaemia of extremities and tissue loss related to vasculitis. The mortality rate in the group that had a CV event was 44% giving an odds ratio for death of 3.8. This mortality risk was especially high for patients with recurrent CV events (odds ratio 8.3). The prevalence of a CV event increased linearly over time leading to a 4-fold risk of mortality. The risk of a CV event increased linearly over time and persistent disease activity was the sole independent risk factor for the first CV event. Treatment with



hydroxychloroquine or antihypertensive medication appeared to have a protective effect.

Having established an increased rate of hard CV endpoints in prospective studies of patients with SLE I will now examine the evidence for an increased prevalence of subclinical CVD in adults with SLE.

### Increased prevalence of subclinical cardiovascular disease

Several groups have used measures of subclinical CVD as surrogate endpoints of CVD. These include measures of structural vascular damage such as carotid artery intima media thickness (cIMT) and measures of calcification of coronary vessels such as multi-detector computed tomography (MDCT) scanning or single photon emission computed tomography (SPECT). They also include measurements of functional change in vessels such as pulse wave velocity (PWV) which measures the distensibility of vessels, and they include measures of intact endothelial response to vasodilation such as flow mediated dilatation (FMD). See table 2.

**Table 2. Surrogate markers for Cardiovascular Disease.**

Name	How it works
<b>Carotid Intima Media Thickness (cIMT)</b>	Ultrasound measurement of the intima and media layer in the carotid artery. Safe, acceptable and reproducible. Measures a structural change.
<b>Pulse Wave Velocity (PWV)</b>	Measurement of the elasticity of large vessels. Can use applanation tonometry or osillometry. Safe and acceptable. Measures a functional change.
<b>Flow Mediated Dilation (FMD)</b>	Measure of endothelial function by measurement of the degree of vasodilation after a period of vessel occlusion. Non-invasive and safe but painful.

<b>Multi-detector Computed Tomography (MDCT)</b>	A 2-dimensional assessment of vessel wall calcium load (usually coronary). Acceptable but radiation burden.
<b>Single Photon Emission Computed Tomography (SPECT)</b>	Measurement of vessel calcium load (usually coronary) utilising a gamma camera. Acceptable but radiation burden.

Using these methods several groups have shown that adults with SLE have a significantly higher prevalence and extent of systemic arterial calcification, (30,31) increased cIMT, (32,33) MDCT calcium scoring, (34) increased PWV (35) and abnormal FMD (36) than age and sex matched controls.

Plazak (34) examined 60 patients before and 1 year after randomisation to atorvastatin or placebo and showed that MDCT calcium scoring and SPECT of the myocardium at baseline and after 1 year showed an increase in coronary calcium deposits in the placebo group. Shang et al (32) measured PWV and cIMT on 32 patients with SLE and 32 controls, showing that SLE was an independent risk factor of sub-clinical atherosclerosis.

Roman and Salmon et al (31,37) measured cIMT in 197 adult patients with SLE and 197 controls, specifically excluding those under the age of 18 years. They showed an increased prevalence of atherosclerosis in patients when compared to controls, with a relative risk of 2.4, interestingly the prevalence was 5.6 times higher among patients younger than 40 years of age. Multivariate analysis, including age, hypertension, diabetes, smoking, cholesterol and lupus status, showed that only age, the presence of lupus and serum cholesterol level were independently associated with atherosclerosis, with the presence of lupus conferring an odds ratio of 4.8. Univariate analysis showed that patients with SLE with atherosclerotic plaque were older, more likely to be white and postmenopausal and had higher systolic blood pressure

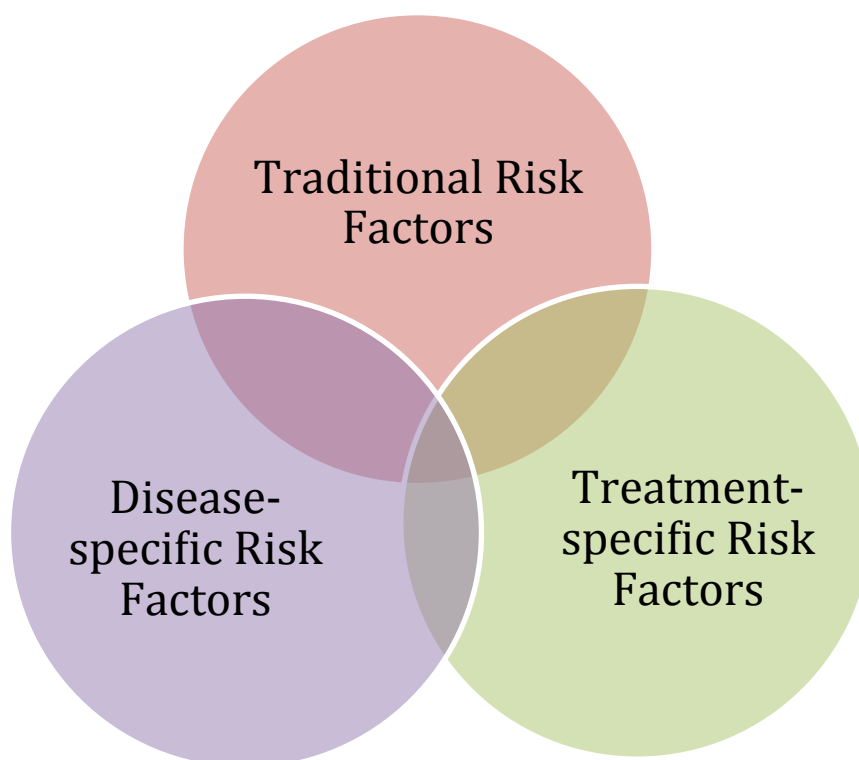
and lower LDL cholesterol than the patients with SLE who did not have plaque. Other factors which independently correlated with atherosclerotic plaque were an older age at diagnosis, longer duration of disease, higher disease activity and not using cyclophosphamide.

Similarly, Yiu et al (30) assessed 50 age matched SLE patients and controls using MDCT to show that patients with SLE have significantly higher prevalence and extent of systemic arterial calcification compared with age and sex matched controls and that in patients with an increased arterial calcium score the most frequent vessel involved was coronary (42%) followed by the carotid artery (24%).

Following on from studies examining structural changes in the vessel wall Yildiz et al (38) measured PWV in 24 premenopausal women with SLE for a mean of 5.3 years and compared them with 24 age and sex matched controls to assess functional status. They found an increase in PWV in patients versus controls ( $8.98 \pm 2.05$  V  $8.05 \pm 0.94$  m/s) showing a decrease in distensibility in women with SLE. This decrease correlated with age, body mass index, waist to hip ratio and systolic blood pressure.

Thus the evidence for an increased prevalence of sub-clinical CVD in adults with SLE is strong, backed up by large prospective population studies and using measures of both subclinical disease and hard clinical endpoints. What is less poorly understood is the pathogenesis of this increased risk.

**Figure 1. Factors Involved in the pathogenesis of cardiovascular disease in systemic lupus erythematosus**



#### **Pathogenesis: Traditional risk factors for CVD in SLE**

Patients with SLE have been shown to have a high prevalence of the traditional CV risk factors, originally identified in the Framingham cohort. Manzi et al (11) examined incidence rates of cardiovascular events in 498 women with SLE in a cross-sectional study compared with the Framingham cohort. Women with SLE were 52 times more likely to have a cardiovascular event, defined as myocardial infarction or angina, than age matched controls from the Framingham cohort. Mean age at first cardiovascular event was 48 years. Mean age of diagnosis of the 498 women was 34 years. An older age at diagnosis, longer disease duration, hypercholesterolaemia, longer duration of steroid use and postmenopausal status were more common in women with SLE who had had a cardiovascular event than those without. This was a well-designed study with a large representative cohort, well-defined end-points and a

suitable control group that reveals a shockingly high rate of cardiovascular events among SLE patients. However, it was a retrospective study.

Later studies have shown lower rates which may reflect improvements in overall treatment, earlier recognition of the signs of CVD or less steroid use in the era of biological agents. More recently, Esdaile et al (39) retrospectively examined SLE registry data from 296 patients and showed that, even after controlling for traditional cardiovascular risk factors, the increase in relative risk was 10.1 for non-fatal myocardial infarction and 7.9 for cerebrovascular accident. Clearly a retrospective study carries a risk of inaccurate diagnoses and this study did not examine softer cardiovascular symptoms such as angina or transient ischemic attacks but nonetheless it highlighted that the increased risk of CVD in SLE could not be explained solely by traditional risk factors. Furthermore Bessant et al (40) examined a large British cohort to compare their predicted risk of CVD, based on traditional risk factors, with their actual rate of CVD and showed that while traditional cardiovascular risk factors predicted the risk of actual CVD in patients over 40-year of age for younger patients their true risk of CVD was higher than that predicted by their traditional risk factors. This study assumed that the control group was free of traditional cardiovascular risk factors and thus may underestimate the excess risk in SLE.

These risk factors are outlined in table 3 and include hypertension, obesity, diabetes mellitus, chronic kidney disease (CKD) smoking, hyperlipidaemia, hyperhomocysteinaemia and a sedentary lifestyle. Not only are these risk factors increased in adult patients but they tend to be clustered in individuals and to interact with disease activity and treatment. For example a patient with very active disease may be treated with significant doses of corticosteroids. The active disease may lead to inactivity and the corticosteroids can have the side effect of increasing

blood pressure, body mass index and impaired glucose tolerance. Santos(41) compared 100 women with SLE, 98 with rheumatoid arthritis and 102 controls and found that modifiable CV risk factors occur more frequently in SLE and rheumatoid arthritis with hypertension and an atherogenic lipid profile occurring more frequently in SLE than rheumatoid arthritis. I will now discuss the prevalence of each of these risk factors along with the evidence that exists for its impact on CVD in adults with SLE.

**Table 3. Candidate cardiovascular risk factors in SLE**

<b>Traditional risk factors for CVD</b>	<b>Potential SLE-specific risk factors for CVD</b>
Increased age	Renal disease
Diabetes	Corticosteroids
Hypertension	Abnormal adipokines
Male gender	Abnormal lipoprotein function
High total cholesterol	Inflammation
High LDL cholesterol	Endothelial activation
Low HDL cholesterol	Disease activity
Family history of CVD	Disease duration
Smoking	Anti-B cell treatment
Reduced physical activity	
Obesity	
Metabolic syndrome	

### Smoking

The prevalence of smoking has been shown in some studies to be higher in SLE patients than in controls and is associated with increased cIMT scores(42). This mirrors the data in healthy adults and large scale population studies such as The Bogalusa Heart Study (43) have shown correlation between increased cIMT and smoking in young adults. Kiani et al (44) examined the vascular phenotype of 187 patients as part of a placebo-controlled trial of atorvastatin. Subjects had MDCT and cIMT measurements twice, 2-years apart. They showed that age was associated with progression of atherosclerosis and after adjusting for age, gender and ethnicity plaque progression was positively associated with smoking but not with measures of disease activity.

### Age and ethnicity

Increasing age positively correlates with both subclinical atherosclerosis and CV events (44,45) in adults with SLE. Kiani et al (44) showed that increasing age was independently associated with the progression of cIMT over a 2 year period, while Sabio et al (45-49) showed that increased PWV was associated with increased age. Salmon et al (31) showed that older age at diagnosis, longer duration of SLE and higher homocysteine concentration are independently related to the progression of AS in SLE.

The presence of carotid plaque has been shown to be higher in African American women with SLE compared with Caucasian women in contrast to studies of non-SLE subjects. (50) Ghosh et al showed increased FMD and cIMT in 60 Indian patients with SLE showing impaired FMD and abnormal IMT.(51) The mean age of the cohort was younger than most studies at  $31 \pm 6$  years), despite this the cIMT was  $0.49 \pm 0.08$ mm in patients and  $0.39 \pm 0.05$ mm in healthy age matched controls. FMD was also impaired in patients ( $23.64 \pm 7.25\%$ ) compared to controls ( $18.97 \pm 7.19\%$ ).

### Gender

The majority of patients with SLE are female and several groups have examined both the impact of oestrogens on the development of SLE and whether males with SLE present a more severe disease phenotype or indeed an entirely distinct disease. In a recent Spanish cohort Alonso et al described a group of 150 adults with SLE, of whom 127 (84.7%) were female.(52) In this cohort women were diagnosed at an earlier age (43 V 54 years) than men, although some commentators have suggested that this may be due to delayed diagnosis in the male cohort.(53) This finding has not been replicated by other groups. A study of 516 adults with SLE in China described 458 females and 58 males. (54) Median age of onset



was younger than the Spanish group at 27.2 years for men and 28.6 years for women.

Several studies have suggested that oestrogens contribute to the onset and development of SLE disease activity.(55) Indeed, one fascinating clinical report linked the development of SLE to exogenous oestrogen therapy in a male to female transsexual.(56) Oestrogen may induce gene expression that leads to immunactivation, perhaps leading to enhanced defence against infection. Peripheral blood mononuclear cells demonstrate enhanced response to immunogen stimulation in the presence of  $17\beta$ -oestradiol. Young et al have shown TLR-8, whose expression is upregulated in SLE, to be an  $17\beta$ -oestradiol responsive candidate gene.(57) Ding et al showed that low serum testosterone was an independent risk factor for the development of LN.(54) Indeed men with SLE seem to have a greater risk of developing LN, whether this is due to delayed diagnosis (53) or less aggressive treatment (54) is not yet clear. Ding et al (54) showed that male patients had a tendency towards higher rates of renal disease (58.2% V 47.2%) with higher SLEDAI scores (16.8 V 12.8). The long term outcome in men with LN appears to be worse. Hsu et al conducted a retrospective chart review of 121 patients with LN, of whom 17 were male, and showed that men were more likely to develop CKD than women. (58) Similarly de Carvalho et al examined the clinical records of 81 adults with LN, of whom 11 were male. They showed that males were more likely to have high creatinine levels (81% V 47%) and a higher renal activity index (7.6 V 4.8). However the frequency of CKD5 and death was similar between the sexes. (59) Wang et al described 415 patients with biopsy proven LN, of whom 45 were male, showing that men had a later diagnosis and worse renal function than their female counterparts. (60)

In summary while there is some evidence to suggest that males present with less musculoskeletal and mucocutaneous disease(61) it is less

certain whether they are more likely to present with severe LN and the evidence suggests that renal outcomes are similar between the sexes.

### Chronic kidney disease

CKD has been described as the “perfect storm” of risk factors for the development of CVD in adults and children (62,63) due in part to mineral bone disease (CKD-MBD) and potentially in part to the treatment of this disorder with calcium containing phosphate binders, vitamin D and dialysis. Even in the early stages of CKD there is a significantly increased risk of a CV event with an adjusted hazard ratio of 2 in CKD stage 2 and 11 in CKD stage 3B, see table 4. (64) Children on dialysis have significantly increased cIMT and PWV when compared to healthy controls with 57% of deaths on haemodialysis and 43% on peritoneal dialysis due to cardiac causes. (65)

**Table 4. The stages of chronic kidney disease defined by glomerular filtration rate.(66)**

Chronic Kidney Disease Stage	Glomerular Filtration Rate ml/min/1.73m <sup>2</sup>	Description
1	>90	Normal kidney function. Known condition leading to kidney disease
2	60 – 90	Mildly reduced kidney function
3	30 – 60	Moderately reduced kidney function
4	15 – 30	Severely reduced kidney function
5	>15	Established/End-stage kidney failure

The UK JSLE Study group(6) has shown that 47% of children have renal involvement at presentation, progressing to 80% at the time of latest analysis as indicated by the pBILAG score (discussed in detail later), but

36% would meet ACR criteria for renal involvement which is higher than their adult equivalents.(14) All children with JSLE are classified as having at least CKD stage 1.

An increase in cIMT and PWV are shown to begin in CKD stage 2-4 (table 4) partly due to dysregulation of the calcium-phosphate-parathyroid hormone axis.(67) Children with pure CKD typically develop arteriosclerosis, rather than atherosclerosis. This is a concentric thickening of the vessel wall with deposition of calcium and phosphate in the tunica media. Over the last decade this has been shown to be a highly regulated process involving the vascular smooth muscle cell. Shanahan et al in 1999 showed that the vascular smooth muscle cell is not terminally differentiated and can differentiate to become adipocytes, chondrocytes and osteoblasts leading to calcification, lipid accumulation and cell death.(68,69)

Fibroblast growth factor-23 (FGF23) is a phosphaturic hormone that contributes to several hypophosphatemic disorders by reducing the expression of the type II sodium-phosphate co-transporters in the proximal tubule of the kidney and by reducing serum 1,25-dihydroxyvitamin D(3) levels.(70,71) FGF-23 requires Klotho, a single-pass transmembrane protein, as co-receptor to bind and activate FGF receptors. Klotho is a senescence suppressor protein that can act as a circulating factor or hormone and, if overexpressed, it leads to increased longevity in mice.(72) Klotho gene mutations are related to endothelial dysfunction, thrombosis and atherosclerosis.(73)

In SLE, proteinuria results in loss of vitamin D binding protein which leads to decreased serum levels of 25-hydroxyvitamin D3 (25-(OH)D) leading to decreased levels of 1,25 dihydroxyvitamin D3 (1,25 (OH)<sub>2</sub>D). This is further exacerbated by reduced 1- $\alpha$  hydroxylation in early CKD. A reduction in circulating 1,25 (OH)<sub>2</sub>D leads to low calcium and phosphate absorption resulting in lower plasma calcium levels. However, activation

of FGF-23 and Klotho leads to decreased tubular phosphate reabsorption, decreased intestinal absorption of calcium and phosphate and the serum phosphate levels are maintained in the normal range. However, as CKD progresses FGF23-Klotho is overwhelmed and plasma phosphate levels rise leading to overstimulation of parathyroid hormone and an increase in serum calcium. Thus maintenance of adequate vitamin D levels early in CKD, along with dietary control of phosphate, may prevent escape of the parathyroid gland from normal control mechanisms.

The greater prevalence of renal involvement in children with JSLE than their adult counterparts suggests that they are at an increased risk of CVD as they age. Early management of CKD in JSLE is a vital part of modifying their CV risk profile. Aggressive treatment of LN appears to have protective effects against premature atherosclerosis, (74) offering some hope that this may be a modifiable risk factor.

### Vitamin D

There is evidence to support a relationship between low vitamin D levels and CVD in the general population, (75) as discussed previously, this is particularly relevant in the context of CKD. The activation of Vitamin D in the body is complex and is likely modified by the presence of inflammatory disease. A recent review by Breslin et al (76) showed a high prevalence of vitamin D deficiency in patients with SLE and this is unsurprising since photosensitivity, sunlight avoidance, urinary loss of vitamin D binding proteins and the use of sunscreens and decreased conversion of 25-(OH)D to the metabolically active 1,25 (OH)<sub>2</sub>D, secondary to renal impairment are known to put SLE patients at risk of Vitamin D deficiency.

Mok examined 290 SLE patients and showed that 78 had vitamin D deficiency. Levels of 25-(OH)D correlated inversely with Systemic Lupus

Erythematosus Disease Activity Index (SLEDAI) score, anti-C1q and anti-dsDNA levels suggesting that 25-(OH)D levels are as specific as antibodies to C1q complement factor (anti-C1q) for detecting concurrent renal activity in SLE. However, the loss of vitamin D binding proteins is a potential confounder in many of these studies and indeed Robinson et al(77) looked at 37 children with JSLE and compared them with 21 patients with juvenile dermatomyositis and found that serum 25-(OH)D levels were inversely associated with disease activity in dermatomyositis but not in non-proteinuric JSLE. See tables 5 – 7 for disease scoring systems.

Studies of animal models and cell cultures show both direct and indirect immunomodulating effects of vitamin D involving T, B and antigen-presenting cells and affecting both innate and adaptive immune responses. Thus the immunomodulating effects of vitamin D may explain the reported epidemiological associations between vitamin D status and a large number of autoimmune and inflammatory diseases.(78)

Polymorphisms in the Vitamin D receptor gene have been associated with the development of nephritis in SLE in certain ethnic groups with Luo et al(79,80) showing that there was a significant difference between the distribution frequencies of Apal and BsmI genotypes in SLE patients versus controls with a significant association between Apal and BsmI gene polymorphism Aa-bb genotypes and the incidence of SLE in the Han population of China. However, Kaleta et al(81) examined BsmI genotype frequencies in 62 Polish patients with SLE and found that no correlation between gene polymorphism and disease activity and no difference between genotype frequencies and healthy controls.

Bogaczewicz et al(82,83) have shown that some patients with SLE to be positive for antibodies to 1,25 (OH)<sub>2</sub>D, the biologically active form of vitamin D which is rarely measured in clinical practice. Wu et al(84) looked at 181 women with SLE and correlated vitamin D levels with CV

risk factors. Lower levels were associated with higher diastolic blood pressure, LDL, lipoprotein (a), BMI and fibrinogen levels.

Low levels of vitamin D remain a potentially modifiable risk factor in patients with SLE, but at this time there is no evidence that supplementing with vitamin D analogues will change the pattern of the disease. More research is needed to advance our understanding of the mechanisms that result in the protective and potentially harmful effects of vitamin D analogues. In particular the effect of vitamin D analogues on vascular smooth muscle cells remains unclear. These cells express both the vitamin D receptor as well as 1- $\alpha$  hydroxylase and 25-hydroxylase enzyme systems and thus vitamin D analogues can have direct effects on vascular smooth muscle cells. Greater understanding of the mechanisms that result in the protective and potentially harmful effects of vitamin D analogues would allow more effective treatment and improved outcomes. (85,86)

## Obesity

An increased prevalence of obesity has been noted in otherwise healthy populations throughout the world and adults with SLE are similarly affected. Up to 50% of adults with SLE are obese (87) whether defined by weight to hip ratio or body mass index (BMI). However, The Nurses' Health Study (12) showed equal age adjusted BMI in the SLE group and the control group (25.4 V 25.6) suggesting that increased BMI is not a pre-existing condition in this large cohort. Certainly individuals with SLE have several potential contributors to their obesity including a reduction in physical activity secondary to fatigue or joint involvement, increased appetite due to glucocorticoid therapy, abnormal adipokines due to chronic inflammation and pre-existing obesity in line with the general population.

Obesity in SLE has been shown to contribute to an increased risk of sub-clinical CVD. Logistic regression analysis of 83 adults with SLE showed that waist circumference was an independent predictor of increased cIMT in a study by Sazliyan et al (88) and Yildiz et al (38) showed a significant correlation between arterial distensibility, as measured by PWV, and age, BMI, and waist to hip ratio.

The impact of obesity is difficult to examine in isolation, and many studies analyse its impact as part of the metabolic syndrome (MetS). It is also impacted upon by physical activity and medications such as corticosteroids, these will all be covered in the following sections.

### Reduced Physical Activity

Adult SLE patients may have lower CV capacity, physical fitness, muscle strength and functional capacity than their peers and when compared with controls matched for age, physical characteristics and physical activity levels and this impacts on obesity and CV health. Katz et al (89) have used the International Physical Activity Questionnaire (iPAQ) to show that 28% of 138 women with SLE and a mean age of 18 years scored less than 600 MET minutes/week which corresponds to a low level of activity.

Similarly, Balsamo et al(90) used the American Thoracic Society protocol (91) to assess the CV capacity adults with SLE compared with controls matched for gender, age and physical activity level, showing a significant difference between the distance walked on the 6-minute walk test ( $598 \pm 45\text{m}$  V  $642 \pm 14\text{m}$ ). However, using the iPAQ which is fully outlined later the methods section, they found that patients diagnosed with SLE were quite active in their daily lives with 92% considered active. In a later study(92,93) the same group found that SLE patients had lower dynamic muscle strength in upper and lower limbs, lower functional performance and greater fatigue than controls. So, despite reporting good physical activity ratings patients with SLE have reduced CV capacity for physical exercise and reduced muscle strength.

Low physical activity scores have been shown to be associated with piHDL and increased subclinical AS in women with SLE,(94). However arterial stiffening was not observed in habitually exercising adults with SLE in a study by Barnes et al (95) suggesting an element of reversibility.



Reduced physical activity may be due to a combination of medication side effects and the underlying disease. Krupp et al (96) have used the Fatigue Severity Scale with 59 lupus patients to show a mean score of  $4.6 \pm 4.5$  out of 7 and this correlated significantly with depression. Similarly Mancuso et al (97) examined 50 adults with SLE and showed that SLE patients who thought that they should be more active walked shorter distances in 2 minutes than those who did not ( $145 \pm 25\text{m}$  V  $170 \pm 39\text{m}$ ) and that patients with more social stress and fatigue reported less physical activity.

The impact of physical activity is important however as several studies have shown its impact on measures of subclinical CVD. Arterial stiffening was not observed in habitually exercising adults with SLE in a study by Barnes et al (95) comparing active adults with SLE, sedentary adults with SLE and healthy controls. Active adults with SLE had similar results to controls whereas sedentary adults with SLE had increased cIMT and decreased ejection fraction. In a later study, Barnes (98-100) used FMD to show, again, similar findings in healthy controls and active patients, and a significantly reduced FMD in sedentary patients compared to controls ( $3.6 \pm 1.3\%$  V  $8.1 \pm 1.2\%$ ) and that this was significantly associated with markers of inflammation and disease activity.

Fatigue is a common feature of SLE and thus patients with mild disease are likely to have lower fatigue scores and may be more physically active. The studies to date (outlined above) appear to show a difference between those who regularly exercise and those who report that they are physically active in their daily lives. As with obesity, however, it is impossible to examine in isolation the role played by physical exercise in increasing the risk of CVD.

### Metabolic syndrome (MetS)

MetS is a known risk factor for CVD. It is defined by the National Cholesterol Education Program Adult Treatment Panel III (101) as three or more of: elevated waist circumference ( $\geq 102$ cm in men and  $\geq 88$ cm in women), hypertriglyceridemia ( $\geq 1.7$ mmol/L), low HDL cholesterol ( $<1.03$  mmol/L in men and  $<1.3$  mmol/l in women), high blood pressure (systolic blood pressure  $\geq 130$ mmHg and/or diastolic blood pressure  $\geq 85$  mmHg and/or pharmacological treatment) and elevated fasting glucose ( $\geq 5.6$  mmol/L and/or pharmacological treatment). Lorenzo et al (101) have shown that it is associated with a significantly increased risk of CVD in adults without SLE. (102)

Clearly the potential impact of prednisolone on each of these risk factors is significant as side effects of corticosteroids include central obesity, hypertension and impaired glucose tolerance. SLE is associated with an increased prevalence of metabolic syndrome and patients have also been shown to have increased insulin resistance. (103-105) MetS has been shown to be associated with an increased CV risk profile in patients with SLE and may contribute directly to accelerated atherosclerosis. (106)

Parker et al(107) studied 200 women with SLE and 100 controls and showed an increased prevalence of MetS in SLE. While Sabio(106) showed that patients with SLE and MetS had a higher PWV and increased biomarkers of subclinical atherosclerosis, such as high sensitivity C-reactive protein (hsCRP) and Interleukin-6 (IL-6), compared to those without MetS in a cross-sectional study.

The Systemic Lupus International Collaborating Clinics Registry for Atherosclerosis inception cohort (108) studied 1494 patients with SLE. All were diagnosed in the previous 15 months, came from 30 centres across 11 countries and had a mean age of 35.2 years, younger than many other registry studies. 239 had MetS at enrolment. Backward,

stepwise multivariate analysis showed an increase in MetS with higher prednisolone dose, Hispanic or Korean ethnicity, current renal disease and immunosuppressant use.

Interestingly unpublished work by Prof Ye Shuang, from the Renji Hospital in Shanghai, presented to the Australian Rheumatology Association Annual Scientific Meeting in May 2014 suggests a role for metformin in the treatment of SLE. Metformin is commonly used in insulin resistance secondary to metabolic syndrome. However, cells treated with metformin *in vitro* showed a 20% reduction in neutrophil extracellular trap (NET) (Knight) formation, along with a decrease in anti-mitochondrial antibody production and a decrease in IFN $\alpha$  production. This *in vitro* work led to a pilot study of 62 patients and 62 controls who were randomly assigned to standard of care or metformin plus standard of care. Mean age of treatment group was 30.7-years, and of the control group was 32.7-years. Metformin was well tolerated with only 3 withdrawals due to adverse events (mild increase in liver function tests (1), nausea (2)). The metformin treated group had a tendency to a reduced number of flares in 6 months, but this did not reach statistical significance. However metformin had a steroid sparing effect and BMI was reduced in the treatment group when compared to controls. This study will be followed up by a larger randomised control trial.

As with many of the traditional risk factors it is impossible to describe in isolation the role played by MetS in increasing the risk of CVD in individuals with SLE, however, it is clearly one of several risk factors, and is potentiated by treatment with corticosteroids.

## Hypertension (HTN)

HTN is a well-established risk factor for CVD and commonly found in patients with SLE with or without renal involvement. In those with renal involvement it may be secondary to sodium retention and fluid overload, depending on the extent of their renal impairment. However even in those without overt CKD excess activation of the renin-angiotensin system and functional nitric oxide deficiency may increase baseline blood pressure.

HTN is also a well-described side effect of corticosteroids, secondary to overstimulation of the mineralocorticoid receptor and thus sodium retention in the kidney, leading to fluid retention and HTN. This leads to a vicious cycle of increased inflammation leading to increased corticosteroids leading to increased blood pressure.

Cyprien (109,110) showed that mean blood pressure was the major risk factor for arterial stiffening in rheumatoid arthritis, as measured by FMD. Since patients with lupus can be hypertensive in the presence or absence of nephritis it is postulated that their underlying disease may be triggering HTN by a unique mechanism that may be responsive to anti-inflammatory medications. More recently, however, researchers have questioned whether HTN itself could be an auto-immune disease, and whether an individual with SLE could produce autoantibodies capable of independently triggering HTN.

A recent study by Lozovoy et al (111) investigated the role of oxidative stress in SLE-associated HTN showed that patients with serologically active disease were significantly more likely to be hypertensive when compared to those with inactive disease and controls. HTN was defined as BP >140/90mmHg or use of antihypertensives. Multivariate analysis showed an association between HTN and an increased Type 1 T-helper cell / Type 2 T-helper cell (Th1/Th2) ratio. This adds to the body of

evidence suggesting that HTN may itself be an inflammatory disease involving the immune system.

Harrison et al (112,113) postulate that this is due to neo-antigen formation and promotion of T cell activation in response to hypertensive stimuli such as angiotensin II, activation of the renin-angiotensin system or a high salt diet. These neo-antigens then promote T-cell activation leading to inflammation in target organs including the kidney and vasculature which manifests as HTN. They have shown that mice lacking T and B cells have blunted hypertensive responses to angiotensin II infusions, normal endothelium-dependent vasodilation and vascular superoxide production but that transplantation of T-cells restores the normal response (114) ie HTN, reduced endothelium-dependent vasodilation and increased vascular superoxide production. Interestingly T-cell modulating agents, such as mycophenolate mofetil (MMF), have been shown in experimental models to prevent renal T cell accumulation and HTN. (115)

### **Pathogenesis: Disease-specific risk factors**

While traditional risk factors may have a role to play in the pathogenesis of CVD in adults with SLE there is also evidence to link disease specific risk factors to its progression. Inflammatory pathways are implicated in increased vascular risk and individuals with chronic inflammatory conditions are known to have elevated risk of CVD. Several studies have shown an increased risk of CVD in inflammatory disease such as rheumatoid arthritis and this appears to be exacerbated in SLE. (41) CV risk in SLE is further complicated by the presence or absence of renal disease, pro-thrombotic risk factors, abnormal lipid profiles and the role played by autoantibodies in initiating endothelial injury and many of these risk factors have been discussed in the previous section.

### Disease duration

Chronic inflammation appears to set in motion a series of events that leads to the initiation and maintenance of atherosclerosis. It makes sense therefore that disease duration would be positively associated with markers of CVD.

The risk of a CV event has been shown to increase with the length of disease. Von Feldt et al (116,117) showed that patients with SLE had higher coronary artery calcium scores than controls in a study of 152 women with SLE and 142 age, gender and ethnicity matched controls and showed that disease duration significantly associated with coronary calcium scores. Plazak et al reviewed 637 patients with SLE over 16-years of age, 43 developed CV damage over a mean disease duration of 6.6 +/-3.6 years. (34) In a 12 year follow up of 156 patients with SLE Becker Merok et al showed that vascular event prevalence increased linearly over time leading to a 4 fold risk of mortality. (29) Rua-Figueroa et al examined 101 adults with SLE on 2 occasions 2 years apart and showed that IMT significantly increases over time and Cyprien showed that endothelial function, measured by FMD, was strongly influenced by duration of disease. Since the duration of disease is correlated with CV risk there is justifiable concern that children, whose disease has an earlier onset, will be at an increased risk as they age.

### Disease Activity

Disease scores for SLE used in clinical practice and research fall into two broad groups. The first are disease activity scores such as the SLE Disease Activity Index (SLEDAI), which gives an overall measure of disease activity, and the British Isles Lupus Activity Group (BILAG), which gives organ specific assessment scales. The second are measures of chronic damage such as the Systemic Lupus International Collaborative Clinics/American College of Rheumatology (SLICC/ACR) which assesses permanent change in an organ system after the diagnosis

of SLE. These scoring systems are outlined in tables 5 – 7. For a diagnosis of SLE using SLICC criteria  $\geq 4$  criteria must be present with at least one clinical and one laboratory criteria or biopsy proven LN with a positive ANA or Anti-DNA.(118,119)

Patients with CV events have been shown in retrospective reviews to have higher disease scores such as SLE Disease Activity Index(SLEDAI)(120-124), and higher Systemic Lupus International Collaborative Clinics (SLICC) scores have been correlated with pre-clinical CV abnormalities,(125,126) such as aortic calcification(94,127-133). Higher SLEDAI scores have also been correlated with lower values for left ventricular longitudinal function measured by strain rate and strain imaging.(126) However even adults with low activity SLE have shown a measureable increase in cIMT over a 2-year period.(134)

CV risk in SLE is further complicated by the presence or absence of renal disease, since CKD is in itself a risk factor for CVD. The prevalence of renal involvement in adult-onset SLE varies with ethnicity from 10% in white patients to 58% in Afro-Caribbean adults with SLE(135) whereas in JSLE it approaches 80%.(6) Proteinuria,(136) elevated serum creatinine(137-139) and a history of nephritis(140) have all been associated with subclinical atherosclerosis in patients with SLE. Prothrombotic factors play an important role in SLE related CVD, with the presence of any antiphospholipid (aPL) antibody or von Willebrand (vWf) factor being independent predictors both of CV events(28,141) and subclinical CVD(142) in the setting of chronic inflammation.

### Antiphospholipid Syndrome

Antiphospholipid syndrome is an important risk factor for CVD in adult-onset SLE. Antiphospholipid syndrome is diagnosed in patients who have vascular thrombosis (arterial or venous) and pregnancy morbidity (foetal death and pre-eclampsia) with persistently positive

antiphospholipid antibodies (anticardiolipin antibodies, lupus anticoagulant antibodies or anti- $\beta$ 2-glycoprotein I antibodies). Although transient nonthrombogenic antiphospholipid antibodies are seen after infections in children, (143) paediatric antiphospholipid syndrome is a rare disease with just 133 patients are registered internationally. (144) 30-40% of adults with SLE have antiphospholipid antibodies but antiphospholipid syndrome complicates only 15% of cases of SLE. The prevalence of antiphospholipid antibodies amongst patients with JSLE may also be influenced by ethnicity. Sixty-two percent of JSLE patients from an Indian cohort (145) were positive for antiphospholipid antibodies while 36 % of the UK JSLE cohort have either levels of anticardiolipin antibodies above the normal laboratory range or positivity for lupus anticoagulant. (6)

Autoantibodies from patients with antiphospholipid syndrome activate endothelial cells (146) and thus potentiate atherosclerosis. Patients with positive antiphospholipid antibodies have been shown to have increased cIMT and PWV, (147) decreased levels of plasma nitrite and impaired endothelium-dependent vascular responses, (148) depleted Tregs (149) and HDL from women with positive antiphospholipid antibodies has reduced nitric oxide bioavailability and impaired anti-inflammatory properties. (148,150) These factors contribute to an increased risk of CVD. MacGregor et al (151) showed that an increase in major vascular disease was only found patients with SLE that had both raised triglycerides and anticardiolipin antibodies.

Currently the management of antiphospholipid syndrome is modification of general risk factors for thrombosis, such as avoiding dehydration, and the use of antiplatelet and anticoagulant agents. The only proven treatment option is long-term anticoagulation with warfarin. (152,153) However, drugs used for other aspects of adult SLE may also modify this condition. Statins block the thrombogenic properties of antiphospholipid



autoantibodies, (154,155) and their multiple effects on monocyte, lymphocyte and endothelial cell activities may contribute to the prevention of thrombosis in patients with antiphospholipid syndrome, although this needs to be validated by larger trials. (156) Hydroxychloroquine has been suggested to reduce the risk of thrombosis, possibly due to the inhibition of platelet aggregation and (157) is associated with lower prevalence of positivity lupus anticoagulant or anticardiolipin antibodies. Furthermore, a small observational study of 32 adult patients with antiphospholipid syndrome and SLE treated with rituximab showed a significant reduction in levels of anticardiolipin antibodies. (158)

Children with JSLE have more severe disease, with a greater prevalence of renal involvement and are consequently likely to be at an increased risk of CVD as they age.

**Table 5. Systemic Lupus International Collaborating Clinics damage index (SLICC)(160)**

<b>Ocular</b>	Any cataract ever	1
	Retinal change or optic atrophy	1
<b>Neuropsychiatric</b>	Cognitive impairment or major psychosis	1
	Seizures requiring therapy for 6 months	1
	Cerebrovascular accident ever (score 2 if > 1)	1(2)
	Cranial or peripheral neuropathy (excluding optic)	1
<b>Renal</b>	Transverse myelitis	1
	Estimated or measured glomerular filtration rate<50%	1
	Proteinuria ≥3.5 gm/24hours	1
	Or end-stage renal disease	3
<b>Pulmonary</b>	Pulmonary hypertension	1
	Pulmonary fibrosis (physical and radiograph)	1
	Shrinking lung (radiograph)	1
	Pleural fibrosis (radiograph)	1
<b>Cardiovascular</b>	Pulmonary infarction (radiograph)	1
	Angina or coronary artery bypass	1
	Myocardial infarction ever (score 2 if > 1)	1(2)
	Cardiomyopathy (ventricular dysfunction)	1
<b>Peripheral vascular</b>	Valvular disease (diastolic murmur, or systolic murmur >3/6)	1
	Pericarditis for 6 months, or pericardiectomy	1
	Claudication for 6 months	1
	Minor tissue loss (pulp space)	1
<b>Gastrointestinal</b>	Significant tissue loss ever (score 2 if > 1 site)	1(2)
	Venous thrombosis or venous stasis	1
	Infarction or resection of bowel, spleen, liver, or gall bladder (score 2 if > 1 site)	1(2)
	Mesenteric insufficiency	1
<b>Musculoskeletal</b>	Chronic peritonitis	1
	Stricture or upper gastrointestinal tract surgery ever	1
	Muscle atrophy or weakness	1
	Deforming or erosive arthritis	1
<b>Skin</b>	Osteoporosis with fracture or vertebral collapse	1
	Avascular necrosis (score 2 if > 1)	1(2)
	Osteomyelitis	1
	Scarring chronic alopecia	1
	Extensive scarring or panniculum	1
	Skin ulceration (excluding thrombosis) for > 6 months	1
	Premature gonadal failure	1
	Diabetes (regardless of treatment)	1
	Malignancy (exclude dysplasia) (score 2 if > 1 site)	1(2)

*\* Damage (nonreversible change, not related to active inflammation) occurring since onset of lupus, ascertained by clinical assessment and present for at least **6 months** unless otherwise stated. Repeat episodes must occur at least 6 months apart to score 2. The same lesion cannot be scored twice.*

**Table 6. British Isles Lupus Assessment Group (BILAG)**

Category		Definition	
A		Severe disease activity requiring any of the following treatment:	
		<ul style="list-style-type: none"> <li>systemic high dose oral glucocorticoids (equivalent to prednisolone &gt; 20 mg/day)</li> <li>intravenous pulse glucocorticoids (equivalent to pulse methylprednisolone ≥ 500 mg)</li> <li>systemic immunomodulators (include biologicals, immunoglobulins and plasmapheresis)</li> <li>therapeutic high dose anticoagulation in the presence of high dose steroids or immunomodulators <u>eg</u>: warfarin with target INR 3 - 4</li> </ul>	
B		Moderate disease activity requiring any of the following treatment:	
		<ul style="list-style-type: none"> <li>systemic low dose oral glucocorticoids (equivalent to prednisolone ≤ 20 mg/day)</li> <li>intramuscular or intra-articular or soft tissue glucocorticoids injection (equivalent to methylprednisolone &lt; 500mg)</li> <li>topical glucocorticoids</li> <li>topical immunomodulators</li> <li>antimalarials or thalidomide or prasterone or acitretin</li> <li>symptomatic therapy <u>eg</u>: NSAIDs for inflammatory arthritis</li> </ul>	
C		Mild disease	
D		Inactive disease but previously affected	
E		System never involved	
<b>Constitutional</b>	Pyrexia	<b>Musculoskeletal</b>	Myositis
	Weight loss		Myalgia
<b>Mucocutaneous</b>	Lymphadenopathy/ Splenomegaly	<b>Cardiovascular and Respiratory</b>	Severe polyarthritis
	Fatigue/malaise/lethargy		Arthritis
	Anorexia		Arthralgia
	Skin eruption		Tendonitis/tenosynovitis
	Angio-oedema		Tendon contractures
	Mucosal ulceration		Aseptic necrosis
	Panniculitis		Pleuropericardial pain
	Digital infarcts		Dyspnoea
	Alopecia		Cardiac failure
	Angio-oedema		Friction rub
	Mucosal ulceration		Effusion
	Malar erythema		Chest pain
	Subcutaneous nodules		Progressive chest Xray changes
	Perniotic skin lesions		ECG evidence of

<b>Neurological</b>	Peri-ungual erythema		pericarditis/myocarditis/endocarditis
	Swollen fingers		Cardiac arrhythmias
	Sclerodactyly		Pulmonary function fall by >20%
			Cytohistological evidence of inflammatory lung disease
	Calcinosis		Myocarditis
	Telangiectasia		Cardiac tamponade
	Splinter haemorrhages		New valvular dysfunction
	Impaired level of consciousness		Pleural effusion with dyspnoea
	Cognitive dysfunction		
	Acute psychosis/delirium/confusional state		Pulmonary haemorrhage/vasculitis
	Seizure disorder		Interstitial alveolitis/pneumonitis
	Status epilepticus		
	Cerebral vascular disease		
	Cerebral vasculitis	<b>Vasculitis</b>	Shrinking lung syndrome
	Aseptic meningitis		Aortitis
	Mononeuropathy		Coronary vasculitis
	Ascending/transverse myelitis		Major cutaneous vasculitis including ulcers
	Demyelinating syndrome		Major abdominal crisis due to vasculitis
	Myelopathy		Recurrent thromboembolism
	Acute inflammatory demyelinating polyradiculoneuropathy		Raynauds
	Peripheral neuropathy		
		<b>Gastrointestinal</b>	Livido reticularis
			Superficial phlebitis
			Minor cutaneous vasculitis
	Cranial neuropathy		
	Plexopathy		
	Polynuropathy		
	Autonomic disorder		
	Disc swelling		
	Chorea		
	Cerebellar ataxia		
	Movement disorder		
	Lupus headache (severe, unremitting)	<b>Ophthalmic</b>	Lupus peritonitis
	Episodic migrainous/tension/cluster headaches		Abdominal serositis or ascites
	Headache from intracranial hypertension		Lupus enteritis/colitis
	Mood disorder (depression/mania)		Malabsorption
	Anxiety disorder		Protein losing enteropathy
	Organic brain syndrome		Intestinal pseudo-obstruction
	Severe hypertension		Lupus hepatitis
	Proteinuria		Acute lupus cholecystitis
<b>Renal</b>	Nephrotic syndrome		Acute lupus pancreatitis
	Histological evidence of active nephritis		Orbital inflammation
			Keratitis
			Anterior uveitis
			Posterior uveitis/retinal vasculitis
			Episcleritis
			Scleritis
			Retinal/choroidal vaso-occlusive disease
			Optic neuritis
			Anterior ischaemic optic neuropathy

**Table 7. Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)(159)**

<b>Wt</b>	<b>Descriptor</b>	<b>Definition</b>
8	Seizure	Recent onset. Exclude metabolic, infectious or drug cause.
8	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, disorganized, or catatonic behavior.
8	Organic Brain Syndrome	Altered mental function with impaired orientation, memory or other intelligent function, with rapid onset fluctuating clinical features
8	Visual Disturbance	Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serious exudate or hemorrhages in the choroids, or optic neuritis.
8	Cranial Nerve Disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8	Lupus Headache	Severe persistent headache: may be migrainous, but must be nonresponsive to narcotic analgesia.
8	CVA	New onset of cerebrovascular accident(s). Exclude arteriosclerosis.
8	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual, infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis.
4	Arthritis	More than 2 joints with pain and signs of inflammation (i.e. tenderness, swelling, or effusion).
4	Myositis	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/adolase or electromyogram changes or a biopsy showing myositis.
4	Urinary Casts	Heme-granular or red blood cell casts.
4	Hematuria	>5 red blood cells/high power field. Exclude stone, infection or other cause.

4	Proteinuria	>0.5 gm/24 hours. New onset or recent increase of more than 0.5 gm/24 hours.
4	Pyuria	>5 white blood cells/high power field. Exclude infection.
2	New Rash	New onset or recurrence of inflammatory type rash.
2	Alopecia	New onset or recurrence of abnormal, patchy or diffuse loss of hair.
2	Mucosal Ulcers	New onset or recurrence of oral or nasal ulcerations.
2	Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
2	Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram confirmation.
2	Low Complement	Decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory.
2	Increased DNA binding	>25% binding by Farr assay or above normal range for testing laboratory.
1	Fever	>38°C. Exclude infectious cause.
1	Thrombocytopenia	<100,000 platelets/mm <sup>3</sup> .
1	Leukopenia	<3,000 White blood cell/mm <sup>3</sup> . Exclude drug causes.
<hr/>		
_____ <i>TOTAL SCORE (Sum of weights next to descriptors marked present)</i>		

### Pathogenesis: Iatrogenic

Since atherosclerosis has been shown to be an inflammatory process it might be hoped that the medications used to reduce inflammation in SLE would also reduce CV risk. Unfortunately in some cases they may independently increase CV risk, acting to increase in traditional CV risk factors such as obesity and HTN. Table 8 outlines the medications commonly used in the treatment of SLE.

**Table 8. Medications used in SLE**

<b>Anti-inflammatory</b>	Aspirin
	Paracetamol
<b>Non-steroidal anti-inflammatory drugs</b>	Ibuprofen
	Naproxen
	Diclofenac
<b>Biguanides</b>	Metformin
<b>Glucocorticoids</b>	Prednisolone
	Methylprednisolone
<b>Antimalarials</b>	Hydroxychlorquine
<b>Immunomodulators</b>	Cyclophosphamide
	Mycophenolate mofetil
	Rituximab
	Belimumab
	Methotrexate
	Azathioprine
	Intravenous immuno-globulin
	Tacrolimus

## Glucocorticoids

The use of glucocorticoids may increase the prevalence of traditional risk factors, such as obesity, hypertension, and impaired glucose tolerance. Some studies have suggested that treatment of SLE with prednisolone may be cardioprotective at moderate doses,(125) but that there is an increased risk of subclinical CVD with low or high doses.(23,161)

It is difficult to tease out the independent effects of glucocorticoids on CV risk. Patients on higher doses may have more active disease, and patients on lower doses may be undertreated, in addition patients with severe disease are likely to be treated with prednisolone in addition to other immunomodulating agents. To compound this confusion, the side effects of glucocorticoids include obesity, impaired glucose tolerance and HTN making it almost impossible to accurately assess their risk and to assess if modification of glucocorticoid regimes would change CV risk.

## Hydroxychloroquine

Originally used as an antimalarial agent, hydroxychloroquine is an inexpensive drug used in SLE, predominantly for cutaneous disease. Its exact mechanism of action is unknown.(162) It is thought to block Toll-like Receptors reducing the activation of dendritic cells. In vitro plasmacytoid dendritic cells from SLE patients treated hydroxychloroquine showed an impaired ability to produce Interferon-  $\alpha$  (IFN- $\alpha$ ) and Tumour necrosis Factor- $\alpha$  (TNF- $\alpha$ ) when stimulated with TLR-9 and TLR-7 agonists.(163) In addition to its efficacy in preventing flares of SLE there is epidemiological evidence(74,88,164) that it may be cardioprotective, possibly by improving the lipoprotein profile(165) and improving vessel elasticity. (166) A recent longitudinal study(167) of 24 patients with SLE treated with hydroxychloroquine showed a reduction in total cholesterol, and LDL after 3 months of therapy.



## Immunomodulators

Cyclophosphamide is an alkylating agent, usually reserved for the treatment of severe SLE-nephritis (LN) and cerebral-SLE. Its mode of action is unclear but it leads to apoptosis and also appears to act on regulatory T-cells. It is a very effective treatment for severe SLE but side effects, including myelosuppression and infertility, limit its use. Roldan showed that individuals treated with cyclophosphamide were 5.4 times less likely to develop atherosclerosis on trans-oesophageal echo. (168,169)

Recent reviews (170,171) have shown that MMF, another anti-T-cell agent, may have equal efficacy as first line therapy for severe LN without the side effect of infertility. Little is known about the effect of MMF on CV risk in SLE but studies of the CV risk profile of post-renal transplant recipients (172) treated with basiliximab, steroids, tacrolimus and MMF or basiliximab, steroids, everolimus (173) and ciclosporine have shown that 6-months post-transplant the second group had greater dyslipidaemia. As mentioned previously (115) animal studies have suggested that MMF may have an independent effect on HTN and thus CV risk it also acts to inhibit VSMC proliferation by inhibition of PDGF secretion by endothelial cells. Mouse models prone to atherosclerosis, such as the Apoe-null mouse, have shown significant decrease in atherosclerotic lesions with MMF treatment.(346) The immunomodulating effects of MMF limit its use as an atherosclerotic agent in the general population but are intriguing in patients with SLE. In mouse models of accelerated atherosclerosis and SLE, MMF decreased the severity of atherosclerosis while improving nephritis and inhibiting the development of autoantibodies. (347) Similarly lupus prone LDLr-null mice treated with MMF a significant decrease in the number of CD4+ T-cells in atherosclerotic lesions despite no change in serum LDL. (348)

Disappointingly however the results in humans have been mixed. Kiani et al examined 24 patients treated with MMF compared with 163 patients who were not and found no difference in coronary artery calcium and cIMT over 2 years, indeed the increase in cIMT was slightly greater in those with high exposure to MMF than those with low, or no exposure. However, this was an observational study and those treated with MMF had lupus nephritis, implying a greater risk of CVD, when compared to those not treated with MMF. (349) While the human data has not been wholly encouraging MMF remains a useful drug for SLE management and the potential of CVD risk reduction further increases its charm.

### Anti B-cell treatments

Assessing the role of B cells in atherosclerosis is complex due to the difficulty in separating the intrinsic biological effects of B cells from the effects of the antibodies they secrete and there is increasing evidence of a variety of functionally separate B-cell subsets. (174) Innate response activator (IRA) B cells develop in the spleen during the inflammatory phase of sepsis and produce granulocyte macrophage colony-stimulating factor (GM-CSF). (175) GM-CSF is known to be atherogenic with deficient mice developing smaller atherosclerotic lesions (176) and exogenous administration leading to larger atherosclerotic lesions. Hilgendorf et al (177) have shown that IRA B cell lines expand in atherosclerosis, increasing plaque size and promoting the generation of dendritic cells.

SLE B-cells are involved in cytokine production as well as the production of autoantibodies which may initiate the inflammatory reaction, they also lead to T-cell activation and thus a medication that targets B-cells has obvious appeal. (178)

Rituximab is a chimeric monoclonal antibody that depletes CD20-positive B cells while sparing stem and plasma cells. Although it is used

widely in the treatment of severe SLE the results of two large-scale controlled trials (179-181) have, disappointingly, failed to demonstrate efficacy. Belimumab is a fully humanised monoclonal antibody against soluble BLYS, a type II trans-membrane protein that functions in a healthy immune response to prolong survival and promote differentiation of B-cells.

Since some animal models demonstrate a protective role for endogenous B-cells in the evolution of atherosclerotic plaque (182) there are concerns about the potentiation of atherosclerosis in the setting of B-cell depletion (183) although, as outlined above they may also be pro-atherogenic.

However, Mathieu et al (184) examined the effect of 24 weeks and 52 weeks of rituximab treatment on CV risk factors, including PWV, lipid profile and BP in 33 patients with rheumatoid arthritis and showed no change in PWV or modification of the lipid profile. Even more encouragingly Gonzalez-Juantey et al (185) showed that FMD improved dramatically in all patients 2 weeks after the first infusion of rituximab in 6 women with rheumatoid arthritis. This improvement was sustained to 6 months and was associated with an improvement in CRP and disease activity scores. Pego-Reigosa et al (186) showed a sustained improvement in both lipid profile and lupus activity in patients treated with rituximab followed for 1 year.

As with all the various therapies used in SLE anti-B cell therapies are generally used in combination with other drugs, especially prednisolone, and may be used after other medications have been trialled, such as MMF. Thus it is difficult to assess their effects in isolation.

## Statins

Statins are a class of drugs which reduce serum cholesterol levels by inhibiting HMG-CoA reductase and thus hepatic cholesterol production

and there is strong evidence that they are effective in preventing the development of CVD in an otherwise healthy population with hypercholesterolaemia. (187) Along with inhibiting cholesterol biosynthesis their anti-inflammatory effects include upregulation of NO synthesis, inhibition of inflammatory cytokine production, decreased monocyte and endothelial cell adhesion, decreased expression of tissue factor by macrophages, inhibition of oxidised-LDL-induced macrophage proliferation, inhibition of ROS formation and reduced T-cell activation. (188) They have a variety of direct effects on gene expression and the function of the innate and adaptive immune systems. This has led some to postulate(189,190) that they could have disease modifying effects in SLE. Moreover, animal studies (191,192) have shown improvements in inflammatory markers and renal disease in murine models of lupus treated with statins.

However, 2 large clinical studies have failed to show any appreciable benefit to treatment with statins in SLE. Petri et al (193) randomised 200 adults with SLE to atorvastatin or placebo for 2 years. Results showed no difference in coronary artery calcium, cIMT, carotid plaque, hsCRP or markers of endothelial activation. In a similar study with a paediatric population, discussed later, Schanberg et al (194) showed no effect of atorvastatin on the progression of cIMT over 36 months, in patients with JSLE. Post hoc analysis showed some benefit in post-pubertal patients with higher hsCRP. The original cohort excluded patients with renal insufficiency or active nephrotic syndrome and thus may have underrepresented disease severity.

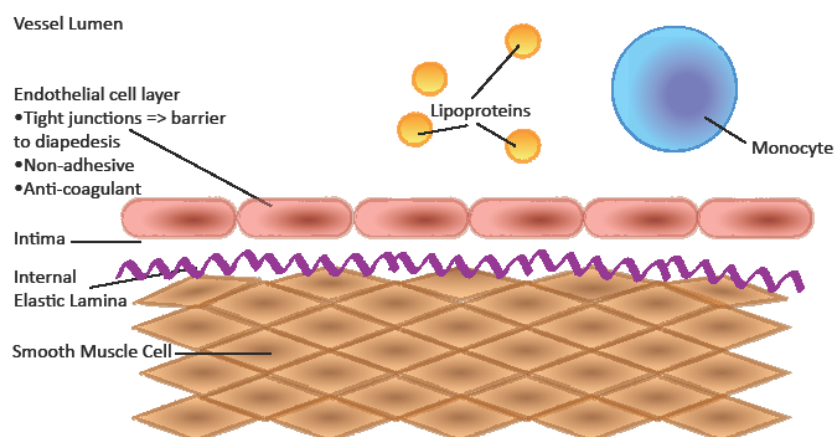
Thus currently adults with SLE and dyslipidemia are commonly treated with statins but they do not currently form part of the routine treatment regimen for children and adolescents with JSLE.

## The pathogenesis of Atherosclerosis in individuals without SLE

Atherosclerosis is a disease of large and medium sized muscular arteries. It is characterized by vascular inflammation, which leads to vascular remodeling and plaque formation. The interactions that lead to the formation and maintenance of an atherosclerotic lesion are not fully understood but the “response to injury” hypothesis (195-197) is the most widely accepted explanation. This hypothesis proposes that endothelial injury leads to vascular inflammation resulting in a fibroproliferative response. In an otherwise healthy individual endothelial injury may result from toxins, such as the by-products of tobacco, hyperglycaemia, oxidized Low Density Lipoproteins (LDL), hyperhomocystinaemia, cholesterol or infectious agents. (198,199) Abnormal activation of inflammatory pathways (34,200,201) by systemic diseases, such as SLE can also initiate atherosclerosis.

Despite the systemic nature of CV risk factors, atherosclerotic lesions occur at areas where there are sudden changes in the velocity of blood flow ie areas of branching and increased curvature. This is likely due to the interaction between mechanical forces and shear-stress responsive elements (202) on the endothelial cell (EC) acting to up- or down-grade physiologically relevant genes leading to increased EC turnover, oxidative stress and increased expression of molecules such as platelet-derived growth factor and vascular cell adhesion molecule-1 (VCAM-1) and resulting in an EC that is primed for the development of AS. (203-206) Figure 2 depicts a healthy blood vessel with an intact endothelial layer.

Figure 2. Healthy blood vessel wall (illustration by C Quinlan)



Injury to the endothelial wall whether resulting from toxins or an imbalance between vascular damage and repair in an inflammatory milieu, permits the accumulation of LDL in the intimal layer of the vessel wall. (42,63,207-209)

**Figure 3. Endothelial inflammation results in monocyte differentiation (illustration by C Quinlan)**

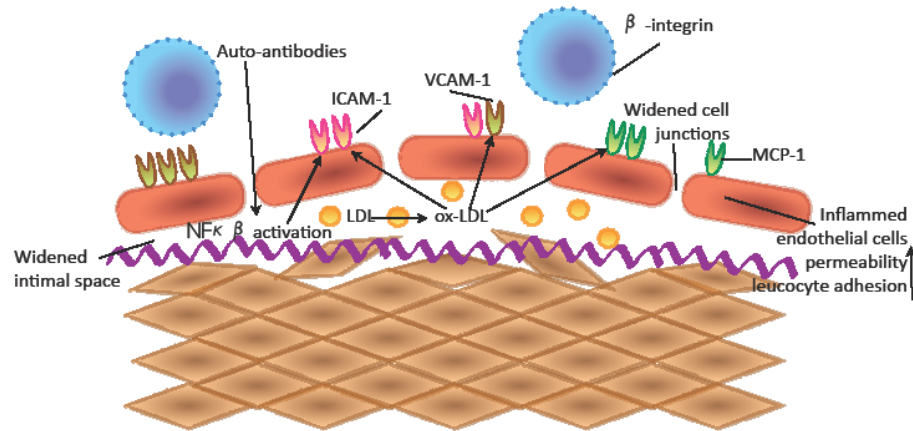


Figure 3 depicts the initial stages of AS, showing endothelial inflammation, widening of the intimal space, increased permeability of the endothelium, EC expression of cell adhesion molecules and the differentiation of monocytes to macrophages.

As LDL moves into the sub-endothelial space it becomes oxidized by various enzymes in the vessel wall including xanthine oxidase and myeloperoxidase. Accumulation of oxidised LDL stimulates the EC to express adhesion molecules, such as ICAM-1, VCAM-1 and MCP-1, which attract and transport monocytes from the vessel lumen, through the endothelium and into the vessel wall.

**Figure 4. Monocyte diapedesis and smooth muscle cell migration (illustration by C Quinlan)**

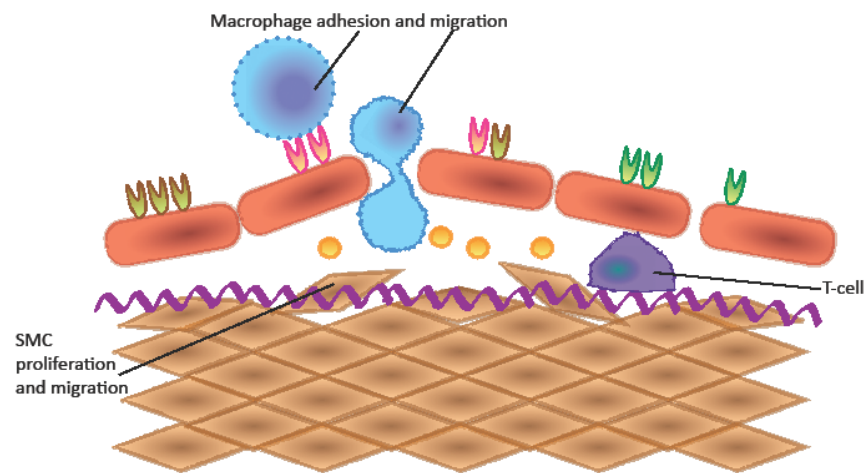
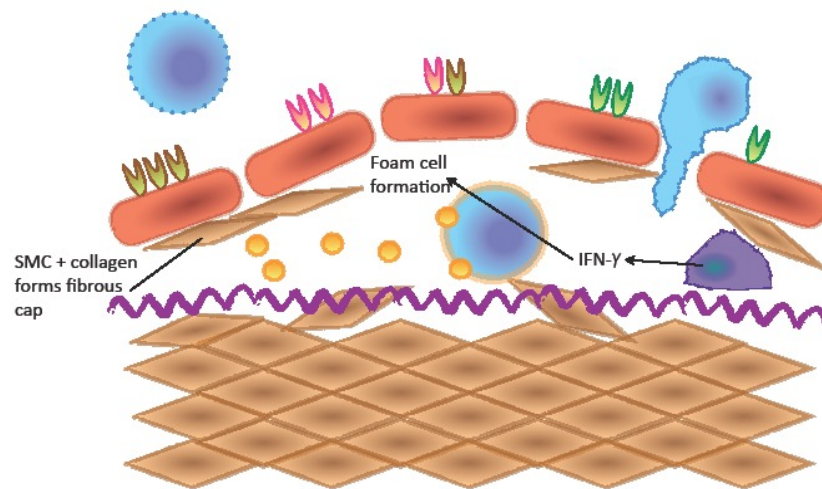


Figure 4 depicts the movement of macrophages into the widening intimal space, the initial migration of vascular smooth muscle cells (VSMC) and the presence of T-cells in the developing plaque.

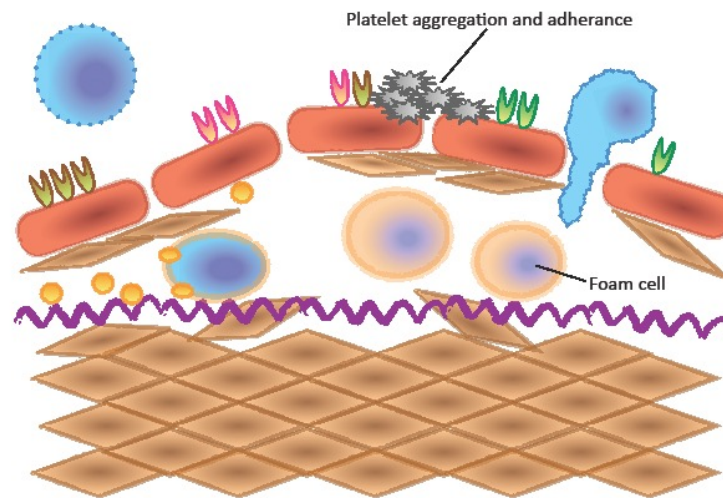


**Figure 5. Oxidised LDL is phagocytosed by macrophages which become foam cells (illustration by C Quinlan)**



Macrophage Colony Stimulating Factor (MCSF) promotes the differentiation of monocytes to macrophages which then phagocytose the oxLDL molecules, becoming lipid-rich “foam cells”.(210-212) An accumulation of these “foam cells” along with T-lymphocytes and smooth muscle cells forms the earliest pathological lesion of atherosclerosis, the fatty streak. These lesions can be found in the aorta from childhood.(213-215) Vascular smooth muscle cells proliferate, migrate and, along with collagen, ultimately form the fibrous cap. This acts, initially, to stabilize the developing plaque and prevent rupture. A fragile microvascular network, the vasa vasorum, supplies the developing plaque, it is prone to haemorrhage which further contributes to the progression of the plaque through the release of inflammatory mediators leading to platelet activation and clumping at the site of the rupture.(216-219) This process is depicted in figures 5 and 6.

**Figure 6. Platelet activation over the site of plaque rupture. Fibrous cap formation (illustration by C Quinlan)**



Interferon- $\gamma$ , produced by T-cells, impairs VSMC proliferation and collagen synthesis, along with matrix metalloproteinases (from the activated macrophages) acts to degrade collagen and weaken the fibrous cap, leading to the rupture of the atherosclerotic plaque.(211,215,220-222) Rupture of the plaque results in thrombus formation, and progression of the plaque due to organization of the thrombus. As the plaque progresses it leads to narrowing of the lumen of the vessel.

**Figure 7. Organisation and potentiation of the atherosclerotic plaque (illustration by C Quinlan)**

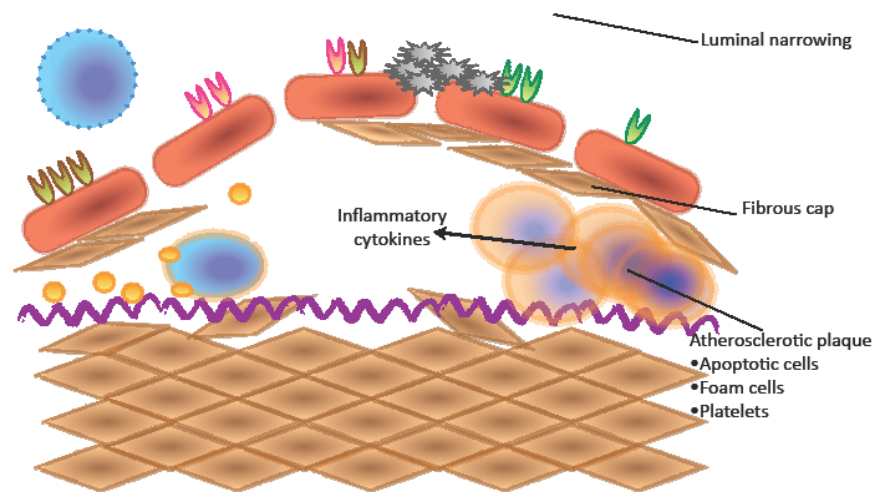


Figure 7 shows organisation of the plaque and highlights luminal narrowing. The atherosclerotic plaque is made up of a mixture of apoptotic cells, foam cells, dendritic cells and platelets, it secretes pro-inflammatory mediators and acts as a nidus for calcification.(223-225)

### Arteriosclerosis

As previously discussed all patients with SLE have at least stage 1 CKD, since they have a condition known to lead to CKD and at least normal serum creatinine. See table 4. CVD is a major cause of mortality in CKD as shown by registry data worldwide. The Australian and New Zealand Dialysis and Transplant registry (ANZDATA) and the Dutch national cohort study have shown that over 50% of deaths on dialysis are due to CVD or cerebrovascular causes.(65,226) CVD in CKD is due to both atherosclerosis and arteriosclerosis.

Arteriosclerosis is a highly regulated process involving promoters and inhibitors of calcification, including FGF23, osteoprotegerin, bone-

morphogenic proteins and Fetuin A(227) leading to phenotypic change in the vascular smooth muscle cell (VSMC).(228) In healthy vessels the VSMC in the medial layer are contractile and allow the vessel dampen down the cardiac impulse and thus prevent shear stress to the endothelial cell layer. However, as CKD progresses the VSMC loses its contractile phenotype and undergoes apoptosis or osteo/chondrocytic differentiation.(229) Apoptosis leads to the release of matrix vesicles containing calcium and phosphate which act as a nidus of calcification.(62,230,231) This process is well established before the clinical signs are evident and recent studies have confirmed that the vessels of children, even in the early stages of CKD, are abnormal.(67) The process is thought to commence in early CKD with FGF-23 the earliest biomarker upregulated. (232) Future cohort studies aiming to recruit children in the early stages of CKD may help to further delineate the underlying mechanisms of arteriosclerosis in CKD.(233)

### **The Pathogenesis of cardiovascular disease in individuals with SLE**

There is some evidence to suggest that the pathogenesis of atherosclerotic plaques in SLE may be in some respects unique.(234) A proportion of adults with SLE with cardiac symptoms do not have plaques on coronary angiogram but may have perfusion defects on myocardial perfusion scanning suggesting mechanisms of ischemia other than plaques(235) such as antibody induced micro-thrombi.(236,237)

### **Dysfunctional endothelial activation**

Endothelial activation is emerging as a key event for accelerated atherosclerosis. An imbalance between vascular damage and repair, possibly induced by interferon, could play a prominent role in the induction of accelerated AS in SLE. (195) Lupus prone NZ black and NZ white F1 mice display endothelial dysfunction and abnormal phenotype and function of endothelial progenitor cells. (182) Recently the endothelial-specific Ang-Tie ligand receptor system has been identified

as a major regulator of vascular responsiveness to inflammatory stimuli. Circulating Angiopoietin-2(Ang2) concentrations have been demonstrated to be increased and concentrations of Ang1 decreased in individuals with active SLE compared to healthy controls. Individual Ang2 concentrations correlate with SLEDAI score, ds DNA titres and sVCAM-1. Ang2 mediated disruption of protective Ang1/Tie2 signalling may be operational in SLE. (238)

In SLE patients, circulating EC counts have been shown to be significantly higher than in healthy controls and strongly correlate with SLEDAI score. The active phase of the disease may be associated with an increased number of circulating ECs (239) and severe SLE flares are characterised by enhanced EC apoptosis. (196,240) The mobilisation of endothelial precursor cells (EPCs) is unaffected in SLE but there is a diminished number, altered phenotype (241) and altered functionality of circulating CD34+/VEGFR2+ cells which may reduce the ability to repair vascular damage and thus may trigger the development of atherosclerosis in SLE.(242)

In SLE auto-antibodies may contribute to the pathogenesis of atherosclerosis by causing injury to the endothelium altering the metabolism of lipoproteins involved in atherogenesis. (36). Risk factors for the development of atherosclerosis in SLE include specific antibodies to beta2GPI, anticardiolipin antibodies, anti-oxidised LDL and antibodies to heat shock proteins, complement activation, impaired ability to activate Transforming Growth Factor-  $\beta$ 1 (TGF- $\beta$ 1), and elevated levels of CRP.(35) Along with this individuals with SLE have been shown to have autoantibodies to Vitamin D, (75,84) HDL (36) and oxidised LDL. (209)

### Abnormal adipokine activity

Many epidemiological and laboratory studies have established obesity as a CV risk factor and it has become evident that white adipose tissue interacts with the vasculature through the secretion of adipokines (243) predisposing the individual to inflammation leading to atherosclerosis. SLE has been shown to be a hyperleptinaemic condition (244) and individuals also show reduced secretion of adiponectin, an important protector of the vasculature. Leptin levels are known to be increased in inflammation and autoimmune disease (241,244,245) Recent work by Vadacca et al exploring the secretion of adipokines in SLE showed raised leptin levels when compared with controls, which correlated with disease activity and measures of abnormal vascular structure. (244,246-248)

Leptin has long been known to modulate bone mass, however more recently it has also been shown to affect VSMC (208,249) proliferation, leading to plaque destabilization and increased thrombosis.(247) It acts on the endothelium to disrupt nitric oxide (NO) production, leading to decreased nitric oxide (NO) mediated vasodilation. Leptin binds with its specific Leptin receptor (LepR), a member of the class I cytokine receptor family widely expressed on different types of cells, and acts as a pro-inflammatory cytokine, leading to increased serum levels of TNF- $\alpha$ , IL6 and IL1. At a functional level leptin polarises T helper cytokine production towards a proinflammatory phenotype. (250) Leptin induced activation of the mammalian target of rapamycin (mTOR) controls leptin production and signalling and stimulates the proliferation of CD4+CD25-FOXP3- effector T cells in vivo and in vitro. (251)

Adiponectin acts to protect the vasculature from atherosclerosis decreasing endothelial cell apoptosis and reducing the adhesion of surface molecules thus reducing the endothelial injury, which can initiate an atherosclerotic plaque. (243,252-255) It defends the VSMC against

injury and decreases VSMC migration, reducing the migration of macrophages and their transformation into foam cells. (241,255) Recent work shows low levels of serum adiponectin in women with SLE, that levels are inversely correlated with measures of vascular function (195,244-246) and that its actions result in increased serum levels of IL1 and IL1RA and decreased levels of TNF- $\alpha$  and IL6. (256)

Adiponectin has 2 different receptors: ADIPOR1 and ADIPOR2. ADIPOR1 is mainly expressed in muscle while ADIPOR2 is expressed mainly in the liver. A small number of T cells express adiponectin receptors (257) on their surface and within vesicles. Indeed, mice deficient in adiponectin had higher frequencies of CD137+ T cells upon infection with Coxsackie B, suggesting that adiponectin may be a novel negative T-cell regulator, (258) and adiponectin has been shown to inhibit allograft rejection in murine cardiac transplantation. (259) This immunomodulator effect could be mediated by its ability to alter dendritic cell functions. (260) In T cells cultured with dendritic cells which had been treated with adiponectin, a higher percentage of CD4+CD25+Foxp3+ Treg cells was seen suggesting that it could control Treg homeostasis. (261) Furthermore adiponectin may be involved in Th1 differentiation (262) inducing maturation and activation of dendritic cells leading to enhance pro-inflammatory responses. (262)

Adiponectin has traditionally thought of as an anti-inflammatory cytokine (263-265) exerting its effect on endothelial cells through the inhibition of TNF- $\alpha$  induced adhesion molecule expression and the inhibition of NF $\kappa$ B activation, and by increasing the secretion of IL-10 and IL-1 receptor antagonist by monocytes, macrophages and dendritic cells and suppressing the production of Interferon- $\gamma$  (IFN- $\gamma$ ) by macrophages and TLR-induced Nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation. However newer studies have shown that it can also act as a pro-inflammatory cytokine. Its levels are high in pre-eclampsia and renal

disease, it induces IL-6 and activates NF- $\kappa$ B in human synovial fibroblasts and adhesion molecule expression in endothelial cells.(266-269)

### Other markers of inflammation

Fetuin A is a negative acute phase reactant made by the liver and a potent inhibitor of calcification. It is associated with an atherogenic lipid profile, (225,270-272) acting to inhibit apoptosis of VSMCs and to protect against their calcification. Low levels appear to impair macrophage function. (256,273,274) It interacts with free fatty Acids to activate Toll-like receptor-4 (TLR4) and increase insulin resistance. (270,275,276) Downstream activation leads to increased levels of TNF- $\alpha$  and IL1. Uncontrolled activation of TLR4 leads to a lupus type autoimmune picture in murine models. It has not been studied in SLE. (277-279)

Interferon- $\alpha$  (IFN- $\alpha$ ) promotes a cytokine profile that enhances anti-angiogenic responses, promotes vasculopathy and accelerates AS. In murine lupus it acts to increase HDL oxidation. (221) It promotes the apoptosis of mature endothelial cells (280) and can affect the megakaryocyte transcriptome and increase platelet activation in vitro. (215) Circulating activated platelets lead to increased atherosclerotic plaque formation. (281) IFN $\alpha$  can induce foam cell formation in vitro. (211) Its expression is increased in areas of atherosclerotic plaque (282) and has been associated with increased markers of subclinical cardiovascular disease, such as increased cIMT. (283)

### T-cells

All T cells begin as CD4-CD8-TCR cells they are produced from progenitor cells in bone marrow, and committed to their lineage in the thymus. Treg cells are a subset of T cells, usually making up 5-15% of peripheral CD4+ T cells in mice and humans. They suppress immune



responses of other cells, maintaining tolerance of self-antigens and thus reducing auto-immunity. CD4+Foxp3+ Tregs are also called naturally occurring Tregs and FOXP3 can be used as a marker. Mutations in the FOXP3 gene in humans is associated with IPEX syndrome , a uniformly fatal X-linked condition characterised by immunodystrophy, polyendocrinopathy and enteropathy.

Taleb et al(250,284) showed that leptin signalling is important in Treg signalling, using a mutant mouse model of combined leptin and Ldlr deficiency to show that leptin deficiency causes reduced atherosclerosis, independent of serum cholesterol levels, and associated with reduced Th1 responses, increased splenic expression of FOXP3 and increased in vitro Treg cell function. Improved Treg cell response was associated with a marked reduction of IFN- $\gamma$  production by CD4+ T cells suggesting inhibition of Th1-mediated pro-atherogenic immunity. Transfer of Treg-deficient lymphocytes and Treg cells from leptin receptor-deficient mice into mutant mice prone to atherosclerosis caused a greater reduction in atherosclerotic lesion size and inhibited more IFN- $\gamma$  production than transfer of Treg cells from wild-type mice.

### Lipoproteins

High Density Lipoproteins (HDL) exert a number of physiological functions, the most studied of which is their ability to promote the transport of cholesterol to the liver for excretion which protects against atherosclerosis. (207,285-287) HDL is a complex particle made up of more than 50 proteins along with phospholipids, triglycerides and cholesterol. In addition to protective proteins, such as apoJ, apoA-I, PON-1, PON-3, and hemopexin, other molecule classes such as complement proteins, endopeptidase inhibitors and pro-inflammatory molecules, such as serum amyloid proteins, are present. Aside from cholesterol, several other substances transported by HDL, such as

apolipoproteins, lipids and enzymes, can modulate endothelial function. (214,288-290)

Many epidemiological and laboratory studies have correlated increased levels of HDL with a reduction in atherosclerosis, primarily through the lipoprotein's role in reverse cholesterol transport from the arterial wall. (214,291,292) However, over the past 20 years alternative protective mechanisms of HDL have emerged, including anti-oxidative, anti-inflammatory, and anti-apoptotic (289,290,293,294) mechanisms which may be altered by chronic inflammation and the ratio of pro-inflammatory : anti-inflammatory proteins in HDL can be changed through post-translational modification or hyperoxidation

The use of statins, such as atorvastatin(34) and rosuvastatin,(295) has been shown to be effective in reducing hsCRP and restraining the progression of atherosclerosis. However in children the evidence shows that statins may slow the progression of cIMT thickening only in a subgroup of pubertal patients with higher hsCRP (296) and does not support its routine use.

More recently attention has focused on alterations in HDL function, brought about by inflammatory states, such as infection or immune mediated disorders, such as SLE. (288) HDL has been shown to prevent the oxidation of LDL and its consequent uptake by monocytes; thus preventing the formation of foam cells, one of the important steps in atherogenesis. This anti-oxidant mechanism is due mainly to the presence of Apolipoprotein A-1 (ApoA1), the major protein fraction in the HDL particle. ApoA1 exerts anti-oxidant properties by stabilising the enzyme paraoxonase, and also has anti-inflammatory properties by blocking the contact mediated activation of monocytes by T cells. Inflammation induces changes in HDL levels and its composition is altered by the binding of acute phase products, such as serum amyloid A and ceruloplasmin. (297-300) Dysfunctional HDL is unable to prevent

the oxidation of LDL that occurs spontaneously in vitro and actually increases oxidation, and thus can be considered pro-oxidant and proinflammatory.

Several studies have reported altered lipoprotein profiles and altered HDL function (41,94,253,293) in SLE, identifying 2 patterns of dyslipoproteinemia in SLE. One is attributable to active disease and consists of reduced HDL levels and apoprotein-A1 with elevated levels of Very Low Density Lipoprotein (VLDL) and triglycerides (TG). The second is mainly attributable to corticosteroid therapy (301) and consists of increased total cholesterol, VLDL and TGs. B-cell depletion with rituximab and cyclophosphamide is followed by an increase in HDL and a fall in the total cholesterol/HDL ratio and TG levels in the majority of patients coinciding with a decrease in disease activity as measured by the BILAG index. (186) Those with active disease have been shown to have higher serum levels of TG and VLDL and lower HDL when compared with those with inactive disease. (302,303)

Interestingly, a study by McMahon et al (293) suggests HDL may undertake a dysfunctional phenotype, actively contributing to AS, under chronic inflammatory conditions. Indeed, Skaggs et al (304) have reported the presence of a pro-inflammatory HDL (piHDL) in 45% of adults with SLE versus 4% of controls, which correlated with carotid artery plaque. PiHDL appears to increase monocyte motility in a similar manner to Low Density Lipoprotein (LDL), influences monocyte transcription, accelerates monocyte migration and increases Monocyte chemotactic protein-1 (MCP-1) and TNF- $\alpha$  transcription. The atherosclerotic phenotype is driven by piHDL in part through its interactions on monocytes through Platelet Derived Growth Factor Receptor- $\beta$  (PDGFR $\beta$ ) -mediated pathways.

Titres of antibodies to HDL have been shown to be significantly higher in patients with acute coronary syndrome, in the absence of autoimmune

disease, these antibodies have also been found in individuals with SLE with a reported frequency of between 7.7 and 32.5%. And they have been shown to be higher in those with persistently active disease, as measured by the BILAG index. (36)

### Juvenile-onset SLE

Children with SLE tend to have a more aggressive disease, with a higher incidence of arthritis, nephritis and neurological involvement. (6) Since disease severity and duration are closely linked to CVD in the adult population it is reasonable to assume that the paediatric population would similarly be at increased risk of CVD. Despite the wealth of data available on CV risk in adults with SLE there is a dearth of information about CV risk in individuals with JSLE and most of our knowledge comes from studies in adults. Although childhood lupus is often a more aggressive disease the greater plasticity of children's vessels may protect them from vascular disease making extrapolations from adult studies difficult. PWV has been shown to be increased in active SLE (305) and others have shown an increase in cIMT and PWV that correlate with disease activity.(306) The 4 studies to date that have directly examined CVD in JSLE are summarised in table 9.

The Atherosclerosis Prevention in Paediatric Lupus Erythematosus (APPLE) trial (140,296,307) assessed cardiovascular risk in 221 patients with JSLE before and after treatment with atorvastatin. cIMT was measured as the mean of measurements taken at 4 angles on the far wall in systole (defined by ECG) using a 5 second digital clip and Image Pro software. PWV was not measured.

65% of patients were Hispanic or non-Caucasian, with 27% identified as Afro-American. Mean age was 15.7 years and 83% were female. Patients had mildly active disease (mean SLEDAI 4.6), 34% had hypertension, 36% had LN, and 25% had proteinuria. Increased cIMT was associated

with increasing age, longer disease duration, minority status, higher BMI, male sex, increased creatinine clearance, higher lipoprotein(a) level, proteinuria, azathioprine and prednisolone treatment. In multivariate analysis moderate dose of prednisolone were associated with decreased cIMT while high and low doses were associated with increased cIMT. (140,307) Patients were randomised to 36 months of atorvastatin or placebo and results showed no statistically significant difference in cIMT progression in the statin treated group. However *post hoc* analysis revealed lower cIMT progression in the post-pubertal treatment group with higher hsCRP suggesting that perhaps this subgroup might benefit from treatment with statins. However this study overall did not show improvement and has not changed treatment recommendations to date. It is unfortunate that a measurement of vascular function, such as FMD or PWV, was not part of the protocol and it is worth noting that this was an ethnically diverse group with mildly active disease, which may explain the lack of significant results for the overall group. The lack of a control group is also a serious criticism of this study as it limited the authors' ability to say if the cIMT results with their method were actually normal or abnormal at baseline.

The Early Determinants of Atherosclerosis in Paediatric SLE trial (308) examined 54 patients using a rigorous protocol including cIMT, FMD and PWV. 31 patients, 11 males, had all the vascular studies. cIMT was measured off-line, using ECG gated images, with electronic calipers. The group showed showed some elevation of arterial stiffness, PWV 8.1m/s, but cIMT (0.43mm), FMD and myocardial perfusion were normal. Cumulative prednisolone dose was shown to be correlated with total cholesterol and elevated LDL and hydroxychloroquine therapy correlated negatively with total cholesterol, LDL and apolipoprotein B. In multivariate analysis LDL correlated with cumulative prednisolone dose and negatively with hydroxychloroquine treatment. Boros et al concluded that an increased burden of traditional and non-traditional risk factors

and early evidence of insulin resistance and increased central arterial stiffness were present in paediatric SLE.

Sozeri et al (306) examined 51 patients with JSLE from Turkey, 13 males, compared with 25 controls. cIMT was measured with electronic calipers, from a single image frozen in diastole and PWV was measured by vicorder. They showed an increase in mean cIMT when compared to controls, 0.54mm versus 0.35mm, and an increase in PWV when compared to controls, 6.56m/s versus 5.29m/s. 20% of patients had hypertension, 8 had left ventricular hypertrophy. 27% had LN. However patients with nephrotic syndrome, renal impairment, and dyslipidaemia were excluded. This study included a comparable control group and thus was able to make proper comparisons and show a difference in cIMT and PWV in children with JSLE.

El Gamal et al (305) used PWV of the proximal aorta to compare vascular function in 30 children with JSLE with 16 controls. Patients were divided into 2 roughly equal groups, active and inactive disease. Disease activity was defined by SLEDAI with 4 patients in the active group having mild or moderate activity and 12 having high or very high activity. Median SLEDAI score for the active group was 17.5 (2 – 71). PWV was measured in the proximal aorta using echocardiography to show an increase in PWV in the active disease group when compared with controls and no difference in the inactive disease group. Absolute values were not given in this paper. This was a good study but unfortunately the PWV measurement method cannot be extrapolated to other studies.

Thus the studies examining CVD in JSLE to date have significant methodological heterogeneity which makes it impossible to compare data between groups. The largest study, APPLE, unfortunately did not include a control group and showed no significant results until subgroup analysis.

**Table 9. Comparison of paediatric studies examining cardiovascular risk in juvenile-onset systemic lupus erythematosus**

Author	Location	N	Patient Characteristics	Surrogate markers for CVD	Pertinent findings
<b>Ardoin</b> (140,296,307)	USA	221	Mean SLEDAI 4.6 33% HTN 33% LN 25% proteinuria	cIMT 0.47mm	Increased cIMT progression in post-pubertal patients with higher hsCRP
<b>Sozeri</b> (306)	Turkey	51	13 male 20% HTN 27% LN	cIMT 0.54mm PWV 6.56m/s	Higher PWV in hypertensive patients  Marginal increase in PWV in LN
<b>El Gamal</b> (305)	Egypt	30	4 male 28 previous proteinuria 16 current proteinuria Active group mean SBP 139mmHg	Aortic PWV measured by echo	Increased PWV in active disease, no difference in quiescent disease
<b>Boros</b> (308)	Canada	54	11 male 14 HTN	cIMT 0.43mm PWV 8.1m/s	No difference to controls

*Cardiovascular disease, CVD; Systemic Lupus Erythematosus Disease Activity Index, SLEDAI; Hypertension, HTN; lupus nephritis, LN; Pulse Wave Velocity, PWV; Systolic Blood Pressure, SBP.*

## Conclusion

There is sufficient epidemiological evidence, from prospective registry studies and retrospective reviews, to show that adult onset SLE is associated with an increased risk of clinical and preclinical CVD. The increased prevalence of traditional risk factors in this cohort, such as obesity, makes it difficult to study the effect of SLE-specific interventions. Many will already have a significant lifetime CV risk due to non-modifiable risk factors such as a lifetime of smoking, metabolic syndrome and age. Additionally SLE-specific risk factors such as disease severity, disease duration, the use of corticosteroids and antiphospholipid syndrome appear to be associated with an increased prevalence of sub-clinical CVD.

Children with JSLE generally have more severe disease and will have a longer lifetime burden of disease. What evidence there is suggests that they may also have an increased burden of CVD. Since their baseline age-related risk of CVD is low, they provide a cleaner model to examine the role that SLE and its treatment play in potentiating CVD. They also provide the opportunity to examine the role that disease-modification may play in reducing their lifetime risk of CVD.



# Methodology

Research describing the pathogenesis of CVD has advanced rapidly over the past decade. Early structural changes to the vascular phenotype have been characterised using ultrasound and CT and correlated with functional changes in the vessels(309,310) and with CV morbidity and mortality.(311,312) Concurrently biomarkers for CV events have been identified such as cardiac troponin levels, fibrinogen, CRP, interleukin-6 and hsCRP. More recently the use of techniques to evaluate the structure of the vessel wall, such as cIMT, and its function, such as PWV, have been used in large-scale population based, prospective studies, such as the Avon Longitudinal Study of Parents and Children (ALSPAC) (147,313,314) and the Adolescent Type 1 Diabetes Cardio-Renal Intervention Trial(315) (AddIT) research group. This has resulted in the development of protocols for their use, resulting in a greater reproducibility between trials.

In this chapter I have discussed the vascular imaging techniques used in the course of this thesis, the selection of subjects, statistics used and the assays performed. I devised the study protocol, obtained ethical approval, recruited the initial 25 subjects, interviewed the subjects, performed and analysed their vascular scans and performed and analysed their adipokine assays. My independent work makes up the majority of this thesis. Professor Jameela Kari continued the program, scanning and interviewing 20 subjects using identical techniques and study proforma. Her work is acknowledged throughout this thesis and in publications arising from this project. I performed the statistical analysis of the entire cohort independently and wrote the papers arising from this work. The routine biochemical, immunological and haematological assays were performed in the clinical laboratories in Great Ormond Street Hospital for Children NHS Trust.

All the work that was not independently performed by myself has been acknowledged in the appropriate sections.

## **Subjects**

JSLE is a rare condition and patients are usually cared for by both paediatric rheumatologists and paediatric nephrologists. This study was performed in four sites across two countries and utilised a combination of basic science and clinical research.

### **Great Ormond Street Hospital for Children NHS Trust**

Children with JSLE were recruited from the nephrology and rheumatology departments in Great Ormond Street Hospital for Children NHS Trust, the largest paediatric centre in the UK caring for children with JSLE. The project was supervised by Dr Kjell Tullus and Dr Stephen Marks, consultant nephrologists in Great Ormond Street Hospital for Children NHS Trust, and was developed in consultation with Dr Clarissa Pilkington, consultant rheumatologist in Great Ormond Street Hospital for Children NHS Trust.

### **The Vascular Physiology Unit at the UCL Institute of Child Health**

The UCL Institute of Child Health in partnership with Great Ormond Street Hospital for Children NHS Trust is the largest centre in Europe devoted to clinical and basic research in children's health. All vascular scans were performed within the Vascular Physiology Unit using protocols and equipment validated previously and used in large scale studies including AddIT and ALSPAC.

### **Trinity College Dublin**

The adipokine assays were performed in The Institute of Molecular Medicine in Trinity College Dublin who kindly provided bench space, equipment and advice.

### **The Children's University Hospital**

Controls for the adipokine assays were recruited from healthy children in The Children's University Hospital, Dublin.

## Recruitment

The UK JSLE Cohort Study has recruited 276 patients in the UK who fulfilled  $\geq 4$  of the ACR SLE criteria and were under the age of 17 years at the time of diagnosis. Approximately 60 children with JSLE are under the care of the nephrology and/or the rheumatology services in Great Ormond Street Hospital for Children NHS Trust and it is the biggest single centre contributing patients to the UK JSLE cohort.

Children with JSLE attending the nephrology or rheumatology services in Great Ormond Street were approached by C Quinlan or J Kari and asked to take part in the study. They were provided with age appropriate verbal and written information and given time to ask questions and decide if they wished to take part in some or all of the study. Informed consent from the parents or adult patients and assent from the children was obtained. See appendix 3.

## Exclusion Criteria

- Children with JSLE who had been diagnosed with end-stage kidney disease (i.e. those on dialysis or post-transplant) were excluded as renal failure is a known major risk factor for CVD and would thus confound the results.
- Children within 6-weeks of a major surgical procedure were excluded as the procedure could affect their inflammatory markers and thus could confound the results.
- Children under 5-years of age were excluded from the study due to concern about their compliance with the vascular studies and a lack of reference information for normal values.

## Control Population

Siblings of children and young adults under the care of the renal or rheumatology team in Great Ormond Street were approached and asked to take part in the full study. However due to poor recruitment (only one control sibling was recruited) a historical control group previously recruited within the Vascular Physiology Unit at the Institute of Child Health was used for comparison of the vascular phenotype. This control data has previously been published and was used with the kind permission of Dr Rukshana Shroff.(316)

A second control group was recruited from healthy children, over the age of 5-years, without chronic underlying disease who were free from infection or inflammation and who had not had surgery in the preceding 6-weeks. They were recruited from the Children's University Hospital in Dublin. These children had demographic and anthropometric data recorded, a serum sample stored and were used for comparison with the adipokine studies. These children were drawn from the emergency department and the GP phlebotomy department without chronic diseases. In the main they were children who presented to the Emergency Department with abdominal pain, which was subsequently diagnosed as constipation, and children whose parents requested a blood test as they looked pale, but who were found to have normal haematological markers. Similar patients were not available in Great Ormond Street.

Although information regarding ethnicity was not formally collected for either control group both are acknowledged to be predominantly Caucasian which limits comparisons. Despite this limitation the use of a control group represents a strength of this study.

## Exclusion Criteria

- Children with an underlying chronic medical condition were excluded.

- Children within 6-weeks of a major surgical procedure were excluded as the procedure could affect their inflammatory markers and thus could confound the results.
- Children under 5-years of age were excluded.

## Ethics

The study was approved by the Institute of Child Health and Great Ormond Street Hospital research ethics committee and The Children's University Hospital, Dublin, research ethics committee. Full details of ethical approval are contained in Appendix 1.

## Assessment of Vascular Phenotype

A rigorous protocol for training in measurement and analysis of cIMT and PWV was developed by the Vascular Physiology unit at the UCL Institute of Child Health for use in multi-site studies with multiple operators to ensure inter-observer reliability. The protocols are outlined in the following sections. The use of this method of training and the protocols for measurement and analysis represent a major strength to this study since they ensure both concordance between both operators, CQ and JK, and will further enable comparison when this group is reassessed in 5-years.

## Measurement of Carotid Artery Intima Media Thickness (cIMT)

Structural changes to the arterial wall occur long before a clinical CV event and over the last 25-years the measurement of the intima and media layers lining the carotid artery using ultrasonography has been established as a surrogate marker for atherosclerosis and CVD.(62,228,316) Paediatric studies have determined normative values(316) and its use in large-scale, population based, cohort studies has resulted in reproducible study protocols. Data for rates of progression exist(317-320) showing that cIMT in adult control groups

increase by 0.006mm/year. Furthermore the measurement of cIMT by ultrasound is safe, non-invasive and acceptable even to young patients.

There are several techniques which have been used to measure cIMT utilising both B- and M-mode ultrasonography. Most studies measure IMT on the far wall of the carotid artery and this has been shown to be more reliable and reproducible (321) than its measurement on the near wall. The site of measurement is equally variable but more recently studies have used a segment 1-2cm proximal to the carotid bulb as it is easy to visualise and thus standardise. In this study cIMT was measured using B-mode ultrasound of both common carotid arteries using a 12-MHz linear-array transducer.(322) using a single ultrasound machine (Vivid7, GE Medical, Horton, Norway) by Catherine Quinlan (CQ) and Jameela Kari (JK).

Identical scanning protocols were used by both CQ and JK and both were trained and certified by the The Vascular Physiology Unit at the UCL Institute of Child Health to perform and analyse these scans. An accreditation process consisting of the measurement of known cIMT in 5 healthy staff volunteers at 2 intervals, 2 weeks apart, ensuring less than 5% variation in individual vascular measures was a requirement of certification. See appendix 2.

### **cIMT Scanning Protocol**

1. As part of the consent procedure the study procedure was explained to both the child and parent using photos to illustrate positioning. See Appendices 3 and 4.
2. The sonographer (CQ or JK) was positioned in a comfortable position at the end of the examination bed, behind the child's head. Figure 8.
3. The child was asked to lie supine, with their head turned at 45° with their neck extended on a pillow and their arms at their sides, in a

temperature controlled room. During measurement of left cIMT the child was asked to slightly turn their head to the right and during measurement of the right cIMT they were asked to turn their head slightly to the left. They were asked to raise their chin slightly. See figure 8.

4. A blood pressure cuff was placed on the upper arm and a 3-lead ECG was attached.
5. Subject details were inputted and saved with the ultrasound cineloop.
6. The carotid bulb was identified in transverse view and the image was saved.
7. The probe was turned through 90° to visualise the common carotid artery in longitudinal view. The view was enlarged and the probe manipulated to find the clearest definition of the lumen-intima and media-adventitia interfaces on both near and far walls over the visible length of the vessel. Occasionally this was not possible, for example due to difficulties with subject positioning or a twisted vessel, in these cases the view was optimised to show the lumen-intima and media-adventitia interfaces on both near and far walls over a 1cm length of the vessel at a distance of 1cm from the carotid bifurcation.
8. The probe was held steady for 15 heart cycles and a cineloop was recorded for off-line analysis. See figure 19.
9. The process was repeated on the contralateral side.
10. The blood pressure was recorded.



## Analysis of cIMT

cIMT is a dynamic measurement and can be measured in different ways. It can be measured in real time with electronic calipers or off line using an automated computerised edge tracking method. Measurements are taken in end diastole which can be defined either by the ECG (on the R wave) or by vessel diameter (at the point of minimum vessel diameter). In this study the measurements were performed offline, using an automated computerised edge tracking software, in end diastole as determined by minimum vessel diameter.

Measurements in end-diastole have been shown by Wikstrand(323) to reduce variability in cIMT measurement. For this study the definition of end-diastole as minimum vessel diameter was used as it has been shown to have greater reproducibility when compared with measurements from ECG-gated frames.(324) All measurements were made in triplicate to reduce measurement error and increase precision.(324,325) Across the 1cm length of artery approximately 120 individual measurements were made, averaged across 3 measurements and resulting in a mean-mean cIMT, an average of 360 measurements which is less sensitive to error or outliers.(325-328)

The same ultrasound machine was used for every scan and the same computer was used for all analysis to maximise standardisation.

Training in vascular scanning techniques took place in the Vascular Physiology Unit at the UCL Institute of Child Health as per the ALSPAC protocol described by Donald et al(213) including the reproducibility assessment. Training in assessment techniques were assessed with analysis of a bank of 25 cine-loops of cIMT whose measurements had previously been determined. These were analysed twice, 2-weeks apart, and the results were required to be both correct and within 5 % of each-

other before accreditation for vascular scanning was granted. In this way both reproducibility and accuracy was ensured.

#### cIMT analysis protocol

1. All images were analysed using Digital Imaging and Communications in Medicine (DICOM) software.
2. Once the image was opened, it was calibrated to determine the pixel size for the vessel diameter measurements, then the cine-loop was played and the section with the clearest boundaries was selected. Figure 10.
3. The region of interest was selected as follows See figure 11:
  - a. Optimal choice was a segment 1cm distal to the carotid bulb measuring at least 0.7cm. The width of this box determined the number of points along the cIMT which will be measured.
  - b. If this was not possible then a smaller segment was selected, which encompassed the clearly defined region, 1cm distal to the carotid bulb.
4. The automatic edge-detection software suggested where the media-adventitia lay, using pink lines, and this was adjusted for accuracy. Figure 12
5. The image was played and observed closely to ensure that the pink lines stuck to the media-adventitia boundary as occasionally echoes within the vessel lumen can lead to inaccuracies.
6. From the image in figure 12 three peaks and troughs were selected. These peaks were chosen as having:
  - a. The most similar morphology

- b. They were adjacent to each-other
  - c. They showed the pink lines staying within the media-adventitia boundary
  - d. Their vessel diameters were within 0.1mm of each-other.
7. Three corresponding troughs to either side of the chosen peaks were selected.
8. The far wall cIMT measurements were recorded on the study worksheet.

**Figure 8. Optimal positioning of patient for cIMT scanning, head placed at 45 degrees, slight chin lift and head supported by a folded towel**

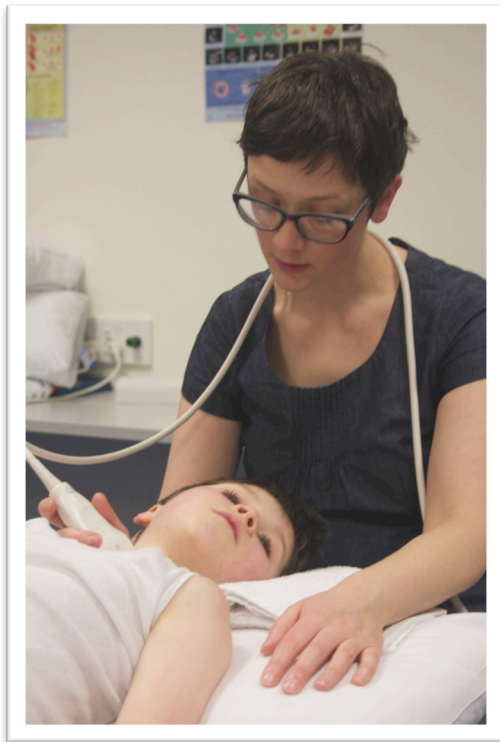


Figure 9. Carotid bulb identified in longitudinal view

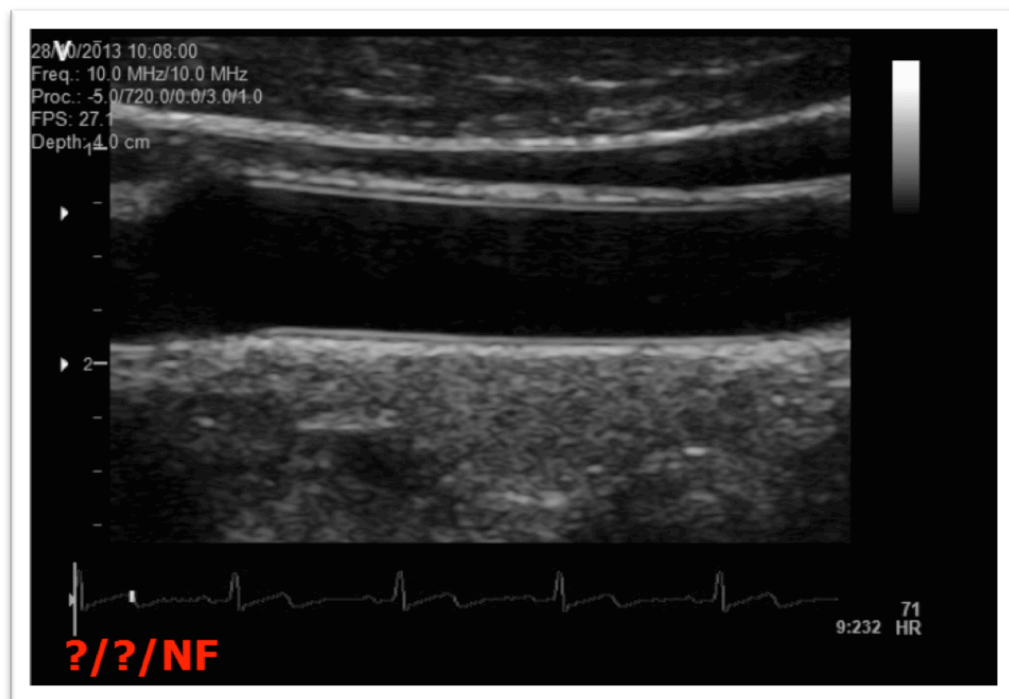


Figure 10. Still image from cine-loop showing clear outlines of lumen-intima and media-adventitia interfaces on the near and far walls over the length of the vessel

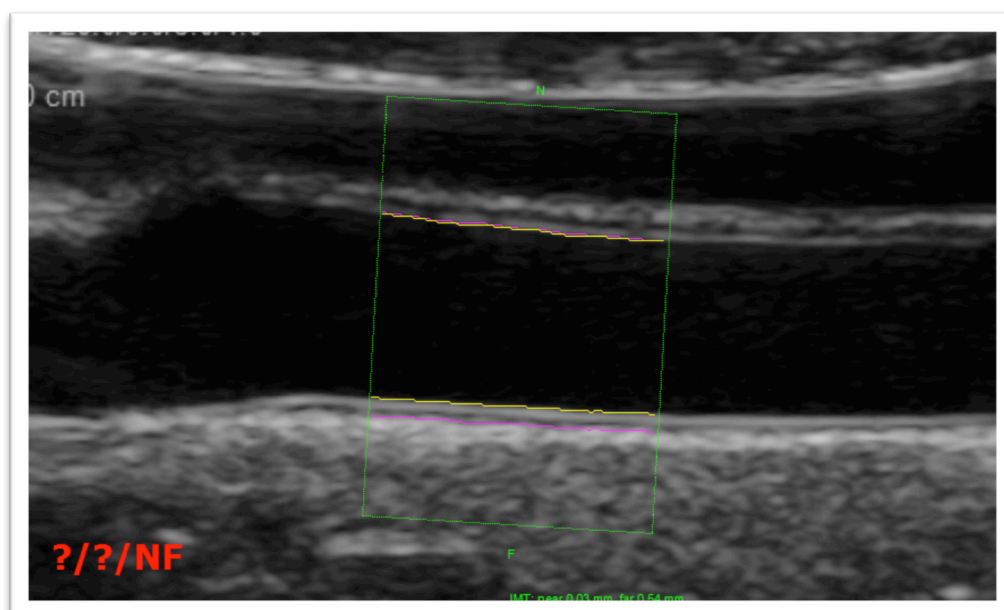
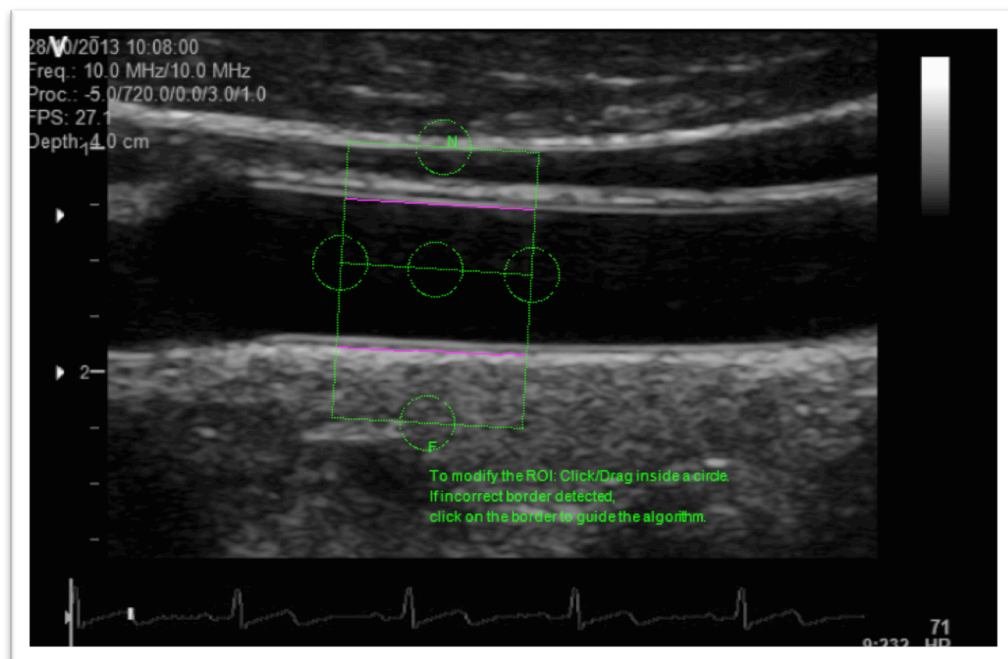
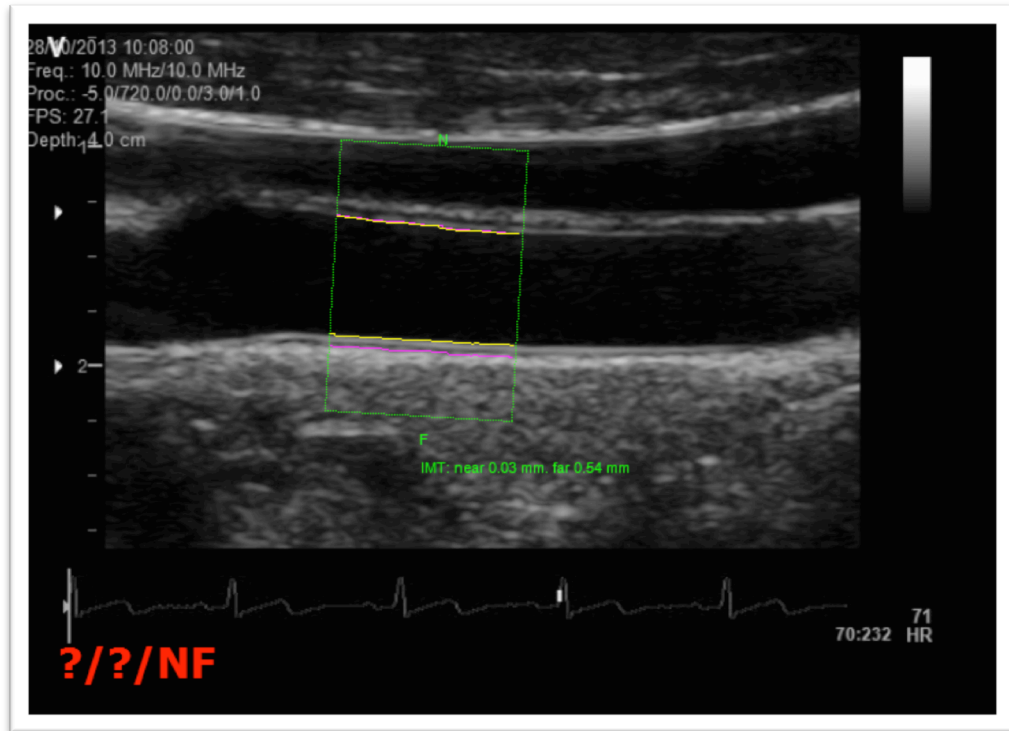


Figure 11. Selection of the region of interest



**Figure 12. Still image from DICOM analyzing software showing the automated edge detection system.**



### **Measurement of Pulse Wave Velocity**

While acting as a conduit for oxygenated blood the elastic wall of the artery also transforms the pulsatile blood flow from the left ventricle into smooth non-pulsatile flow in capillaries. The ability of the artery to dampen down this pulsatility depends on the compliance of the vessel wall. Stiffer, less compliant arterial walls will result in a more rapid pulse wave velocity (PWV). A high PWV results in a greater blood pressure reaching the end arterioles, potentially leading to damage of vulnerable capillary beds, and compromises cardiac perfusion.(329) The assessment of arterial stiffness using PWV is recommended for adults by The European Guidelines on Cardiovascular Disease Prevention(330) and for children by the American Heart Association Atherosclerosis,

Hypertension and Obesity in Youth Committee of the Council on Cardiovascular Disease in the Young. (95,110,120,213,233,331,332)

Carotid-Femoral PWV is a direct measurement, which includes the aorta and large elastic arteries, and thus is likely to have more clinical relevance than Carotid-Radial PWV as changes in the aorta precede those in the brachio-radial arteries. There are multiple ways to measure PWV, including mechanotransducers, ultrasound, computerised oscillometry and applanation tonometry. In children the majority of normative data has compared applanation tonometry with oscillometry. At the time of writing oscillometric measurements, such as the vicorder, are gaining in popularity and are being incorporated into the protocols of large longitudinal studies, such as the 4C study.(233) Recent data has shown good correlation between the vicorder and the more validated applanation tonometry measures.(120,124) However, at the time of formulating the study protocol the gold standard was held to be applanation tonometry, with the Sphygmocor one of the best validated(333) and thus this was used as part of the study protocol.

At the time the study protocol was designed it was unclear which anatomical markers should be used for measuring the distance between the carotid and femoral pulse points i.e. direct (the distance from the carotid pulse point to the suprasternal notch + the distance from the suprasternal notch to the femoral pulse point) or indirect (the distance from the carotid pulse point to the suprasternal notch + the distance from the suprasternal notch to the femoral pulse point via the umbilicus) (see figure 15). Thus both measurements were recorded. At the time of writing the evidence(124,316) lies in favour of using the indirect measurement and thus this is reported throughout this study.

The disadvantage to applanation tonometry, such as the Sphygmocor, is the difficulty in learning the scanning technique and the reproducibility of its results. By contrast, the oscillometric methods, such as the



Vicorder, are easy to learn, have a much less steep learning curve and good inter-observer reproducibility.(233) However, in the Vascular Physiology Laboratory at the UCL Institute of Child Health there is a rigorous training and accreditation program designed to minimise intra-observer variability, both CQ and JK were trained and accredited through this program. Following training PWV was measured in five individuals whose correct measurements were known to the department twice, 2-weeks apart. These measurements were required to be both accurate and within 5% of each-other in order for the scanner to be certified.

PWV is defined as pulse wave travel distance divided by the time difference between the rise delay of the distal and proximal pulse, as measured by the R wave on the ECG. The SphygmoCor system uses a pressure sensor over the carotid and femoral arteries to record sequential pulse waves and measures the time delay between the foot of the pulse pressure arriving at the carotid and femoral arteries. It uses a tonometer, ECG leads and non-invasive blood pressure measurements. SphygmoCor software measures the delay from the R-wave on the ECG to the foot of the pressure pulse by subtracting the R-wave from the pulse foot time at the proximal and distal sites. PWV is calculated from the arterial length and transit time using the intersecting tangents algorithm. (120-124) (334) The software automatically measures the PWV and the operator can be confident about the result if the waves are consistent with each-other and with that which is expected at that pulse point.

It is a non-invasive measurement, which is acceptable to patients or study subjects. A mean of 3 readings was taken, where the child was able to remain still for 3 recordings.

An accreditation process for PWV measurement and analysis was also undertaken as described above and in the ALSPAC protocol described by

Donald et al(314) including the reproducibility assessment. This involved scanning of 5 healthy staff volunteers on 2 occasions, 2 weeks apart and ensuring less than 5% variation in individual vascular measures.

#### PWV measurement protocol

1. The study subject was asked to rest for 10 minutes in a supine position in a quiet dark room. In practice this was usually during the cIMT measurement.
2. The ECG leads were attached and the subject's blood pressure was measured.
3. The subject's details, including blood pressure, were entered into the SphygomCor software.
4. The position of the strongest carotid pulse was palpated and then the tonometer was placed on the pulse point, see figure 13. The tonometer was manipulated to obtain good waveforms. This data was saved on the laptop and the measurement point was marked on the skin.
5. The position of the strongest femoral pulse was palpated and then the tonometer was placed on the pulse point. The tonometer was manipulated to obtain good waveforms. This data is saved on the laptop and the measurement point was marked on the skin.
6. This process was repeated 3 times where the patient was able to keep still.
7. The upper edge of the supra-sternal notch is palpated and marked with the skin marker.
8. The direct (the distance from the carotid pulse point to the suprasternal notch + the distance from the suprasternal notch to

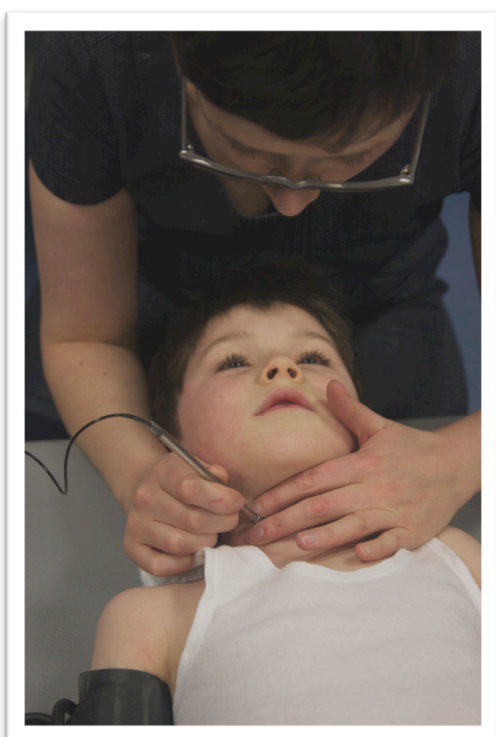
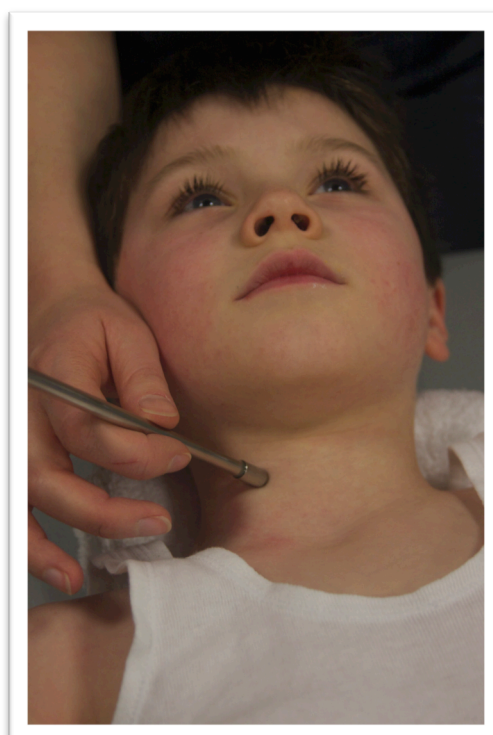
the femoral pulse point) and indirect (the distance from the carotid pulse point to the suprasternal notch + the distance from the suprasternal notch to the femoral pulse point via the umbilicus) measurements were made using a tape measure on the subject's skin. Figure 14.

9. The direct and indirect measurements were entered into the software and both measurements were calculated.

10. The data was evaluated for quality control.

- a. The pulse waveforms should have sharp inflections so that the foot of the wave is easily identifiable.
- b. All the waveforms should be consistent.
- c. The standard deviation of the timings (ms) should be <6%
- d. The Standard deviation of the PWV value should be <10% of the PWV value.

Figure 13. Measurement of PWV



**Figure 14. Measurement of distance from carotid to femoral pulse points**



## **Data Collection**

### **Questionnaire**

A proforma questionnaire (Appendix 5) was used to collect patient information in order to maintain consistency between CQ and JK. A pilot version of the questionnaire was trialled with 10 patients in The Children's University Hospital, Dublin. Following this, it was altered for usability and was used by CQ and JK to record demographic data, treatment information and cardiovascular risk factors. Patient data was anonymised, by means of a randomly generated 4 digit study identification number, and entered into an excel spreadsheet.

## Serology

Routine blood samples were processed by the Departments of Haematology, Immunology and Clinical Chemistry at Great Ormond Street Hospital. Results were recorded by the main researcher. These routine samples were:

- Full Blood Count (FBC)
- Liver Function Test (LFTs)
- Renal Function Test (UEC)
- Parathyroid hormone (PTH)
- Thyroid Function tests (TFTs) – Thyroid stimulating hormone, T3, T4
- Cholesterol
- Triglycerides
- Lipoproteins (LDL/VLDL/HDL)
- ANA
- Anti-dsDNA
- ENA
- C3
- C4
- ESR
- Urine Albumin to Creatinine ratio (UaUc)
- Anti CD19 if clinically indicated – ie if treated with rituximab/belimumab

- Anti cardiolipin antibodies (AC)
- C-reactive protein (CRP)
- High sensitivity CRP (hsCRP)
- C1q
- AntiC1q (C1QA)
- Immunoglobulins – IgG, IgM, IgA
- Serum Ferritin

Serum for storage was left to settle at room temperature for 1 hour. Then spun and divided into 0.5ml aliquots and labelled with the date, subject initials and the study identification number. They were then stored at -21° for 1-2 days and subsequently transferred into the -80° freezer for longer-term storage.

Similarly the urine sample was spun and divided into 1ml aliquots and labelled with the date, subject initials and the study identification number. They were then stored at -21° for 1-2 days and subsequently transferred into the -80° freezer for longer-term storage.

Anti-cardiolipin antibodies were ordered for each subject but as this was an unfunded study the test was rejected by the GOSH laboratory as “not clinically indicated” for 27 patients. These results were not included in the analysis.

### Anthropometry

Body Mass Index (BMI) and centiles were calculated using the Centres for Disease Control and Prevention BMI-for-age growth chart as described by Flegal and Cole.(335) See Appendix 6.

### Clinical information

A positive family history for cardiovascular disease was defined as per the Expert Panel on Integrated Guidelines for Cardiovascular health and Risk Reduction(126) as a CV event in a male relative before 55 years or a female relative before 65 years. Family history included siblings, parents, uncles, aunts, grand-parents, grand-aunts and grand-uncles.

Estimated glomerular filtration rate (eGFR) was calculated by the Schwartz formula with a K value of 34, as calculated locally.(336) An initial attempt to collect cumulative steroid data was foiled by inadequate and inaccurate clinical records.

### pBILAG-2004 Score

Many of the children recruited to this study were co-recruited to the UK JSLE Cohort Study.(6) As part of the protocol the paediatric adaptation of the 2004 British Isles Lupus Assessment Group (BILAG) disease activity index was measured 3-monthly and this was included where available, see table 5. The BILAG index was developed as an intention to treat index and has previously been validated in a UK paediatric cohort(337) and shown to be more sensitive than the SLE Disease Activity Index (SLEDAI)-2000 to detect active disease.(338) It is an ordinal scale index with 9 systems (Constitutional, Mucocutaneous, Neuropsychiatric, Musculoskeletal, Cardiorespiratory, Gastrointestinal, Ophthalmic, Renal and Haematological) which divides disease activity into 5 categories; A represents very active disease; B represents moderate disease; C represents mild stable disease; D represents previous disease; E represents no current or previous disease. A continuous total score (pBILAG-2004 score) has been developed and validated(339) and was used in this study to represent systemic disease burden. The use of a single disease activity assessment could be a limiting factor in this study. Atherosclerosis and the development of increased CV risk is a process, the use of a single disease activity assessment could potentially under-



or overall its significance. However, the pBILAG score is very well validated and recognises the presence of previous disease and thus was considered adequate for this study.

### Calculation of Physical Activity

The International Physical Activity Questionnaire (IPAQ) (94,127-133) was used to estimate the physical activity of the study cohort, see appendix 7. The short version of this questionnaire was chosen, as it is a well-developed, internationally recognized instrument, which enabled the scoring of the cohort by Metabolic Equivalent of Task (MET). This questionnaire proposes three levels of physical activity; “low” where no activity or less than 600 MET minutes/week are recorded, “moderate” where 600 – 1500 MET minutes/week are recorded and “high” where more than 1500 MET minutes/week are recorded. This scoring protocol has been validated in adults with SLE.(89)

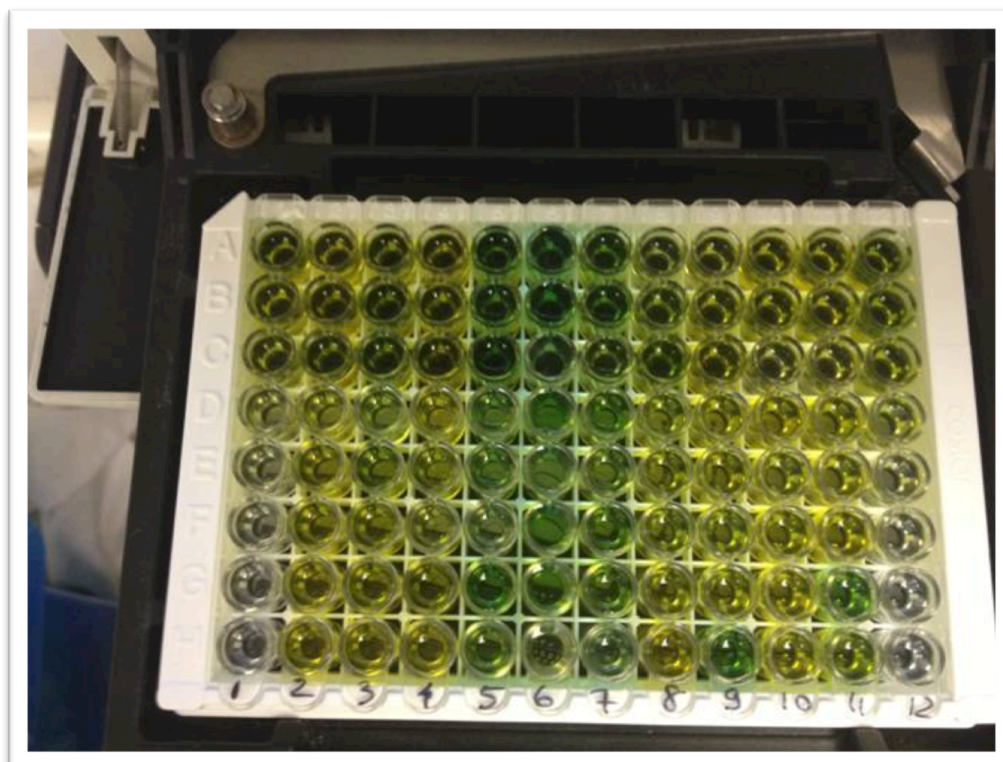
### Measurement of adipokine levels

#### Leptin

Serum leptin levels were determined by a commercial ELISA kit (Human Leptin Immunoassay kit, catalog number DLP00, from R&D systems). Product information sheets are included in Appendix 8. The assay was performed in the Institute of Molecular Medicine in Trinity College Dublin.

The assay used a quantitative sandwich immunoassay technique. The plates were pre-coated with a monoclonal antibody specific for leptin. Standards and samples were added to the wells and the antibody bound any leptin present in the sample. This was then bound by an enzyme-linked monoclonal antibody specific for leptin, resulting in a colour change proportionate to the amount of leptin present when a substrate solution was added to the wells. The intensity of the colour was then measured using a microplate reader. Figure 15.

Figure 15. Leptin/adiponectin ELISA setup



### Leptin ELISA Protocol

1. Reagents and standards were prepared:

The leptin standard (10ng or recombinant human leptin in a buffered protein base) was reconstituted with 1ml of distilled water producing a stock solution of 10,000pg/ml.

The standard was then sequentially diluted with diluent, a concentrated buffered protein base, to the following concentrations: 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml and 15.6pg/ml.

2. 100µL of assay diluent, a buffered protein base, was added to each of the 96 wells precoated with a mouse monoclonal antibody against leptin.
3. 100µL of standard, control or sample was added to each well and the plate was incubated for 2 hours at room temperature. Each sample was analysed in duplicate.
4. Each well was aspirated and washed four times with a wash buffer solution.
5. 200µL of leptin conjugate, a mouse monoclonal antibody against leptin conjugated to horseradish peroxidase, was added to each well and the plate was incubated for 1 hour at room temperature.
6. Each well was aspirated and washed four times with a wash buffer solution.
7. 200µL of substrate solution, made up of equal parts stabilised hydrogen peroxide and stabilised chromogen (tetramethylbenzidine), was added to each well and incubated for 30 minutes at room temperature protected from light.
8. 50µL of stop solution, 2 N sulphuric acid, was added to each well.

9. The optical density of each well was immediately determined using a microplate reader set to 450nm with wavelength correction.
10. A standard curve was created using excel software to generate a log/log graph with the mean absorbance of each standard on the y-axis and the concentration on the x-axis.
11. The concentrations of the samples were read from the standard curve, adjusted for dilution, and the mean values were recorded in an excel datasheet.

### Adiponectin

Serum levels of adiponectin were also determined by a commercial ELISA kit (Human Total Adiponectin/Acrp30, Immunoassay kit, catalog number DLP00 from R&D systems). Product information sheets are included in Appendix 8. The assay was also performed in the Molecular Medicine Unit in Trinity College Dublin.

The assay used a quantitative sandwich immunoassay technique. The plates were pre-coated with a monoclonal antibody specific for adiponectin. Standards and samples were added to the wells and the antibody bound any adiponectin present in the sample. This was then bound by an enzyme-linked monoclonal antibody specific for adiponectin, resulting in a colour change proportionate to the amount of adiponectin present when a substrate solution was added to the wells. The intensity of the colour was then measured using a microplate reader, figure 15.

### Adiponectin ELISA Protocol

1. Reagents and standards were prepared:

The adiponectin standard (500ng of recombinant human adiponectin in a buffered protein base) was reconstituted with 2ml of calibrator diluent (21ml of buffered protein base) producing a stock solution of 250ng/ml.

The standard was then sequentially diluted with diluent, a concentrated buffered protein base, to the following concentrations: 125ng/ml, 62.5ng/ml, 31.2ng/ml, 15.6ng/ml, 7.8ng/ml and 3.9ng/ml.

2. 100µL of assay diluent, a buffered protein base, was added to each of the 96 wells pre-coated with a mouse monoclonal antibody against adiponectin.
3. 50µL of standard, control or sample was added to each well and the plate was incubated for 2 hours at room temperature. Each sample was analysed in duplicate.
4. Each well was aspirated and washed four times with the provided wash buffer solution.
5. 200µL of adiponectin conjugate, a mouse monoclonal antibody against the adiponectin globular domain conjugated to horseradish peroxidase, was added to each well and the plate was incubated for 2 hours at room temperature.
6. Each well was aspirated and washed four times with a wash buffer solution.
7. 200µL of substrate solution, made up of equal parts stabilised hydrogen peroxide and stabilised chromogen (tetramethylbenzidine), was added to each well and incubated for 30 minutes at room temperature protected from light.

8. 50µL of stop solution, 2 N sulphuric acid, was added to each well.
9. The optical density of each well was immediately determined using a microplate reader set to 450nm with wavelength correction.
10. A standard curve was created using excel software to generate a log/log graph with the mean absorbance of each standard on the y-axis and the concentration on the x-axis.
11. The concentrations of the samples were read from the standard curve, corrected for dilution, and the mean values were recorded in an excel datasheet.

## Statistics

Based on control data (n=30) generated in the Vascular Physiology Department in Great Ormond Street by Dr Rukshana Shroff, using iPWV as our primary outcome measure, a group of 23 children with SLE was calculated to provide a 90% power to detect a significant change between controls and children with SLE. Assuming an expected difference between groups of 0.8m/s (ie 15% increase in iPWV) and a mean Standard Deviation (SD) of 0.84 in the primary outcome variable.

For normally distributed data, results are reported as mean  $\pm$  SD and comparisons between means were made using unpaired T test results (2-tailed). For non-normally distributed data, results are reported as median  $\pm$  Interquartile Range (IQR) and comparisons between medians were made using the Mann-Whitney test. Correlations were made using Pearson's correlation co-efficient.

Within this project a p value  $< 0.05$  was determined to have reached significance. Data was entered into an excel spreadsheet as described previously. Statistical analyses were performed in GraphPad Prism version 6.0b for Mac OS X and multiple regression analysis was performed using GraphPad InStat version 3.10 32 bit for Windows (GraphPad Software, La Jolla, California, USA, [www.graphpad.com](http://www.graphpad.com)).





# Clinical Phenotype of Children with Juvenile-onset Systemic Lupus Erythematosus

## Introduction

In this chapter I will examine the clinical phenotype of this cohort. I will describe demographic information, disease severity and the prevalence of traditional or modifiable risk factors for CVD. In doing so I will examine my first hypothesis that children with JSLE have a low prevalence of the classical modifiable risk factors for CVD, obesity, inactivity and smoking, and thus represent a “clean” population in which to study the effect of SLE and the treatment of SLE on the vascular phenotype. This information will also be used in subsequent chapters to identify clinical features associated with the development of an abnormal vascular phenotype.

In the general population, children would be expected to have a low baseline prevalence of traditional risk factors for CVD, such as obesity and smoking, and children with newly-diagnosed JSLE are not different. Thus an integral part of examining their CV risk is establishing their baseline CV profile.

The UK JSLE Cohort Study was founded by the UK JSLE study group, established in 2006. The stated aims of the UK JSLE Cohort Study are to: facilitate hypothesis driven research, determine the demographics of JSLE, determine prognostic markers, assess the validity and reliability of a paediatric version of BILAG-2004, undertake comparative studies with other lupus cohorts, set standards of care of the management of patients with JSLE, develop a cohort of patients for recruitment to clinical interventional trials, facilitate international collaborative studies and trials and perform descriptive analyses of disease presentation, activity damage and response to medication.

The UK JSLE data collection and data repository has previously been described by Watson et al(6) and involves prospective recruitment of patients whose JSLE symptoms began before the age of 17-years.

Demographic data, details of medications, parental and physician global assessments of disease activity and pBILAG-2004 scores are recorded.

The UK JSLE Cohort Study has previously published their review of 198 patients with JSLE and their data is summarised in table 8 adapted from the paper by Watson et al.(6) Of 198 patients, 168 were female, the mean age at diagnosis was 12.6-years and at analysis was 17.4-years. 103 were Caucasian, 29 were Black Afro-Caribbean, 18 were Indian and 12 were Pakistani. With regards to renal function 7(3.5%) had proteinuria and 1(0.5%) had an eGFR <50%. Of the 176 children who had a pBILAG-2004 recorded 141(80%) scored A – D in the renal domain.

The protocol for this study was developed in consultation with the UK JSLE Cohort Study group and thus comparison was made between group demographics to determine if the finding from the smaller CVJSLE Study could be extrapolated to the larger UK JSLE Cohort Study.

## Results

### Demographics

45 children and young adults were recruited to this study, 39 were female. The mean age of the children was  $115 \pm 42$  months at diagnosis and  $161.8 \pm 35$  months at recruitment. Full demographic details of patients and controls are outlined in Table 10. Thirty five children were also recruited to the UK JSLE Cohort Study and 30 had pBILAG scores calculated.

**Table 10. Demographic, clinical and anthropometric characteristics of children and young adults with JSLE and controls.**

	<b>Children with JSLE (n=45)</b>	<b>Controls (n=40)</b>
<b>Age (years)</b>	14(12.2-15.5)	13.2±4.8
<b>Female</b>	39	19
<b>Weight (kg)</b>	50.5(36.5-62)	
<b>Height (cm)</b>	150.1±16	
<b>BMI</b>	21.63(18.19-24.57)	
<b>BMI centile</b>	65.63±28.8	
<b>Biopsy proven lupus nephritis</b>	24	
<b>Disease duration (months)</b>	39(20.5-64.5)	
<b>Age at diagnosis (months)</b>	115 ±42	
<b>Cigarette smoking</b>	0	
<b>Oral contraceptive pill</b>	1	
<b>Positive family history of CVD</b>	11	
<b>Current Prednisolone mg/kg</b>	0.07(0-0.23)	
<b>No prednisolone</b>	16	
<b>Normotensive without anti-hypertensive medication</b>	26	
<b>Enalapril</b>	12	
<b>Amlodipine</b>	8	
<b>Atenolol</b>	2	
<b>Furosemide</b>	2	
<b>Irbesartan</b>	1	
<b>METS/week</b>	1773(676.5-2854)	
<b>Sitting minutes/day</b>	505±144.2	

All figures mean ± standard deviation or median (inter-quartile range)

## Disease Phenotype

Median disease duration was 39 (20.5 – 64.5) months. Disease activity was assessed using the pBILAG-2004 score within 3 months of recruitment. The median pBILAG-2004 score was 3.5 (1.25 – 5.75), in line with the treated national cohort group, with a median pBILAG-2004 score 12 months after diagnosis of 2 (1 – 4).

Renal function is outlined below but renal involvement was also assessed using the pBILAG-2004 score for those who had a score determined and summarised in table 11. 1 child was diagnosed with severe renal disease activity (A), 5 with moderate renal disease activity (B), 5 with stable renal disease activity (C), 16 with currently quiescent but previously active renal disease (D) and only 3 of the 30 children were defined as no renal involvement ever (E).

**Table 11. Renal involvement on pBILAG-2004 disease activity score.**

		N(%)
<b>A</b>	Severe renal disease	1 (3)
<b>B</b>	Moderate renal disease	5 (17)
<b>C</b>	Stable renal disease	5 (17)
<b>D</b>	Previous renal disease	16 (53)
<b>E</b>	Never renal disease	3 (10)

Serology was processed on the majority of children and the results are summarised in table 12. Of those measured, 82% had a positive anti-dsDNA, 57% had raised ESR, 18% had low C3 and 40% had low C4.

**Table 12. Serology for lupus activity**

	<b>Total Measured  N=45</b>	<b>N (%)</b>
<b>Positive anti-dsDNA (&gt;10iu/ml)</b>	28	23 (82)
<b>Raised ESR (&gt;15mm/hr)</b>	35	20 (57)
<b>Low C3 (&lt;0.75mg/dL)</b>	34	6 (18)
<b>Low C4 (&lt;0.12mg/dL)</b>	33	13 (40)

### **Ethnicity**

19 children identified themselves as Afro-Caribbean. Of these, 13 identified themselves as being African or of African descent, 2 identified themselves as Black British, and 4 identified themselves as Black Caribbean. 13 identified themselves as Caucasian. 10 identified themselves as Asian: 5 as Indian, 1 as Pakistani 1 as Chinese and 3 as “other Asian”. 1 child was Afghani, 1 was Turkish Cypriot and 1 declined to answer this question. See table 13 for a breakdown of ethnicities.

**Table 13. Ethnicity of total overall CVJSLE cohort**

<b>Ethnicity</b>	<b>N (total 45)</b>	<b>%</b>
<b>Afro Caribbean</b>	19	42
<b>Caucasian</b>	13	29
<b>Indian</b>	5	11
<b>Other Asian</b>	3	7
<b>Pakistani</b>	1	2
<b>Chinese</b>	1	2
<b>Afghani</b>	1	2
<b>Turkish Cypriot</b>	1	2
<b>Not Stated</b>	1	2

These findings are different to the overall UK JSLE Cohort as outlined in table 14 where the majority of children were Caucasian, and almost a third were Black Afro-Caribbean. By contrast, the CV JSLE Cohort has 42% Black Afro-Caribbean and 29% Caucasian, reflecting the ethnic diversity of London when compared to the rest of the United Kingdom.

**Table 14. Comparison of patient demographic data from UK JSLE Cohort Study and the CVJSLE study(6)**

		<b>UK JSLE</b>	<b>CV JSLE</b>
		<b>N (%)</b>	<b>N(%)</b>
<b>Total</b>		196	45
<b>Gender</b>	<b>Female</b>	168 (85)	39 (87)
	<b>Female:Male</b>	5.1:1	6.5:1
<b>Age</b>	<b>At diagnosis (years)</b>	12.6	9.6
	<b>At analysis (years)</b>	17.4	13.5
<b>Ethnicity</b>	<b>Caucasian</b>	103 (52)	13 (29)
	<b>Black Afro-Caribbean</b>	29 (15)	19 (42)
	<b>Other Asian</b>	12 (6)	3 (7)
	<b>Indian</b>	18 (9)	5 (11)
	<b>Pakistani</b>	12(6)	1(2)
<b>Anti-Hypertensives</b>	<b>ACE-inhibitor</b>	48 (24)	12 (27)
	<b>ARB</b>	24 (12)	1 (2)
<b>Renal Function</b>	<b>pBILAG A-D</b>	141 of 176 (80)	25 of 28 (89)
	<b>Proteinuria</b>	7(3.5)	12 (27)
	<b>eGFR &lt;50%</b>	1 (0.5)	0



## Medications

19 children were not on prednisolone at the time of the analysis. Median prednisolone dose was 8.75 (5 – 15)mg/kg/day. 9 children had been previously treated with rituximab, 1 had just commenced treatment with belimumab and 8 had been previously treated with cyclophosphamide. Numbers were too small to determine differences between groups.

## Traditional CV risk factors

No children admitted to cigarette smoking and only one child had (1 month previous) been commenced on the oral contraceptive pill.

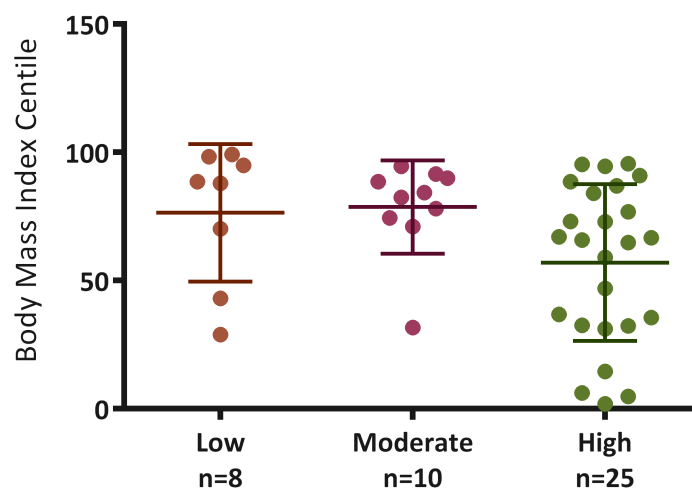
10 children had a positive family history of CVD as defined by Expert Panel on Integrated Guidelines for Cardiovascular health and Risk Reduction and described in the methodology chapter.

Physical activity was scored using the iPAQ (see appendix 7) and expressed in Metabolic Equivalent of Task (MET). “Low” where no activity or less than 600 MET minutes/week are recorded, “moderate” where 600 – 1500 MET minutes/week are recorded and “high” where more than 1500 MET minutes/week are recorded. Median physical activity score was 1773 (676-2854) METs, which correlated with duration of disease ( $r^2=0.325$ ,  $p=0.038$ ). This corresponds to a high level of activity but there was significant heterogeneity within the group. 26 children were in the high activity group. One boy scored 0 METS and admitted to spending all his waking hours sitting to eat or play computer games. In contrast, one girl scored 7146 METS. She was training to be a Physical Education teacher and spent almost all her waking hours engaged in vigorous exercise. Of particular note, the process of examining activity patterns appeared to be of clinical benefit. The boy who scored 0 METS informed his clinician at the follow-up appointment that he had significantly increased his physical activity.

Interestingly, children with a positive family history of CVD had a higher median MET score than children with no family history of CVD (1738 (677 – 2741) V 568 (0 – 1689), but this did not reach statistical significance.

Median BMI was 21.63 (18.19 – 24.57) with a mean BMI centile of  $65.63 \pm 28.8$ . For the control group median BMI was 21.7 (18.3 – 26.7) with a mean BMI centile of  $83.38 \pm 23.37$ . The difference between BMI centile in controls and patients was significant,  $p=0.0051$ . Not surprisingly there was a correlation between BMI centile and physical activity. Median BMI centile for children with a low MET score, ie  $<600$  MET, was 88.2 (49.9 – 97.5) and for those with a high MET score, ie  $>1500$  MET, it was 65.8 (32.5 – 85.5), this did not reach statistical significance, ( $p=0.118$ ). See figure 16.

**Figure 16. Comparison of body mass index centiles in patients with juvenile-onset systemic lupus erythematosus by low, moderate and high activity levels on the international physical activity questionnaire**



## Lipids

Mean serum cholesterol was 4.4 (3.95-5.2)mmol/l, serum triglycerides were 1.04 (0.76-1.93)mmol/l, serum HDL was 1.28±0.46mmol/l, LDL was 2.35 (2.14-2.99)mmol/l and VLDL was 0.64±0.4556mmol/l. Table 15. Abnormalities in serum lipids were correlated with duration of disease, younger age of diagnosis, mean cIMT, PWV, eGFR, increased systolic blood pressure (SBP) and increased BMI. Those with a serum cholesterol greater than 5mmol/L (n=9) had a lower mean daily prednisolone dose of 0.26±0.27mg/kg when compared to those with a serum cholesterol less than 5mmol/L (n=36) who had a mean daily prednisolone dose of 4.28±5.9mg/kg, p=0.054.

**Table 15. Biochemical characteristics of children and young adults with JSLE**

	Children with JSLE (n=45)	Abnormal results	N
eGFR (ml/min/1.73m <sup>2</sup> )	103.3(93.8-113.2)	< 90 ml/min/1.73m <sup>2</sup>	5
hsCRP (mg/L)	0.57(0.29-1.6)	>1mg/dL	5
Cholesterol (mmol/l)	4.4(3.95-5.2)	> 5mmol/L	9
Triglycerides (mmol/l)	1.04(0.79-1.93)	> 2mmol/L	8
HDL (mmol/l)	1.28±0.46	< 1mmol/L	8
VLDL (mmol/l)	0.45(0.33-0.83)		
LDL (mmol/l)	2.35(2.14-2.99)	> 3.5mmol/L	4
ESR (mm/hr)	30.7±26.09	>6mm/hr	25
Urine albumin:creatinine ratio	2.95(1.15-55.33)	>3.5mg/mmol	18

All figures mean ± standard deviation or median (inter-quartile range)

### Anti-hypertensive medications

16 (35.6%) of children were on medication to control hypertension, however, in many of these cases they were treated with Angiotensin Converting Enzyme Inhibitor (ACE-inhibitors) and angiotensin receptor antagonists, likely chosen as much to minimize proteinuria as to control hypertension. Tables 16 and 17 outline the anti-hypertensive medications in use, highlighting the use of enalapril, as well as the use of multiple anti-hypertensives. All patients were normotensive at the time of the study. 26 were not taking any anti-hypertensive medication, 15 were on a single medication (enalapril (9), amlodipine (4) furosemide (1) and irbesartan (1)), 2 were on 2 medications, 1 was on 3 medications and 1 patient required 4 medications to maintain normal blood pressure. 12 children were treated with enalapril, 8 with amlodipine, 2 with frusemide, 2 with atenolol, 1 with doxazocin and 1 with irbesartan.

**Table 16. Anti-hypertensive medications in use for 19 patients**

Drug	N (%)
Amlodipine	8 (18)
Frusemide	2 (4.4)
Atenolol	2 (4.4)
Irbesartan	1 (2.2)
Enalapril	12 (26.6)

**Table 17. Number of anti-hypertensives in use for each patient**

Number of anti-hypertensives in use	N (%)
0	29 (64)
1	15 (33)
2	2 (4.4)
3	0
4	1 (2.2)

### Renal Function

Median eGFR was 103.3 (95.65-113.3)ml/min/1.73m<sup>2</sup>. 5 children had an eGFR < 90 ml/min/1.73m<sup>2</sup>. These were 26.3, 34.5, 66.8, 84.6 and 87.2 ml/min/1.73m<sup>2</sup>. eGFR was correlated with PWV (r<sup>2</sup>=0.331, p=0.055) and abnormalities in serum lipids (r<sup>2</sup>=0.338, P=0.085). 24 children had a history of biopsy proven LN.

A normal UaUc is <3.5mg/mmol and an elevated reading suggests early renal dysfunction or inflammation. Median UaUc for the JSLE cohort was 2.9 (1.2-55.3) mg/mmol. In an effort to assess the effect of renal dysfunction on the vascular phenotype I examined only those children with a moderately elevated UaUc, greater than 20mg/mmol and compared their vascular phenotype with the group as a whole and those with a UaUc closer to the normal range, full characteristics are outlined in table 18.

**Table 18. Demographic, clinical and anthropometric characteristics of children and young people with JSLE, normal and grossly elevated UaUc**

	<b>Children with JSLE (n=45)</b>	<b>UaUc &lt;20mg/mmol (n=33)</b>	<b>UaUc &gt;20mg/mmol (n=12)</b>
<b>UaUc (mg/mmol)</b>	2.9(1.2-55.3)	1.7(0.95-3.5)	164.6(55.3-304.9)
<b>Age (months)</b>	162±35	160±38	165±20
<b>Gender (♀:♂)</b>	39:6	29:4	10:2
<b>BMI</b>	21.6(18.2-24.6)	21.3(18.2-24.3)	22.29(18.7-25.2)
<b>BMI centile</b>	65.63±28.8	63.7±29	70.5±28.8
<b>Disease duration (months)</b>	39(20.5-64.5)	22(21.8-71)	36.5(7.5-49.5)
<b>Positive family history of CVD</b>	11 (24.4%)	9 (27.3%)	2 (16.6%)
<b>Treatment for hypertension</b>	16 (35.5%)	9 (27.3%)	8 (66.7%)
<b>METS/week</b>	1773(676.5-2854)	2190(1127-3306)	886(615-1648)

All figures mean ± standard deviation or median (inter-quartile range)

The groups were similar to the overall cohort with regards to age, gender, disease duration and BMI. Unsurprisingly, the children with elevated UaUc were significantly more likely to be concurrently treated with anti-hypertensive medication, most commonly ACE-inhibitors, used as much for the management of proteinuria as for the management of hypertension. Interestingly, they were less likely to have a positive family history of CVD 16.6% V 27.3%. They were also less active, with median METs of 886 (615 – 1648) compared to those with normal or near normal UaUc levels, 2190 (1127 – 3306), p=0.013.

## Discussion

The hypothesis tested in this chapter was that children with JSLE have a low prevalence of the classical modifiable risk factors for CVD and thus represent a “clean” population in which to study the effect of SLE and the treatment of SLE on the vascular phenotype.

This study has shown no difference in BMI centiles between patients and controls, a low prevalence of sedentary behavior and no smoking. Indeed in contrast to their adult counterparts(89) this cohort of children with JSLE had high levels of activity with a median of 1773 METs per week, corresponding to a high level of physical activity. Encouraging an active lifestyle during clinical encounters is one way in which paediatricians could potentially reduce their patients’ future burden of CVD. However, they have a significant disease burden with a high prevalence of hypertension and renal disease. Thus would be expected to have a significantly increased risk of CVD.

When compared to the UKJSLE cohort study the ethnic background of this group is significantly different. Table 14 shows a reversal of the Caucasian:Afro-Caribbean ratio between the 2 groups. This most likely reflects the ethnic diversity of the catchment area of GOSH, situated in the heart of multi-ethnic London. This ethnic difference must be borne in mind, however, when deciding if findings from this cohort can be generalised to the UK cohort as a whole.

The fact that this cohort is drawn from a tertiary paediatric referral centre probably also explains why the CVJSLE cohort is younger at diagnosis than the UKJSLE cohort with a mean age at diagnosis of 9.6 years for the CVJSLE cohort and 12.6 years for the UKJSLE cohort, again this limitation may affect the generalizability of these results.

Sixteen of the 45 children were taking at least 1 anti-hypertensive medication, most commonly enalapril. This would be prescribed as both

an anti-hypertensive and to reduce proteinuria and the children on an anti-hypertensive were evenly split between low and moderate proteinuria. Table 18. It proved impossible, from the clinical data, to delineate between those who were started on an ACE-I for proteinuria and those who were started on an ACE-I for hypertension thus rendering it impossible to separate these proven risk factors for CVD.

Overall this group has a significant burden of active disease with high inflammatory markers and a high prevalence of renal involvement. This, despite good activity levels and lower BMI scores, will most likely result in an increased prevalence of sub-clinical CVD. In my next chapter I will investigate the vascular phenotype of this group to determine if they have abnormal vascular markers of CVD.



# **Vascular Phenotype of Children with Juvenile-onset Systemic Lupus Erythematosus**

## Introduction

This cross-sectional study was designed to establish the baseline CV risk of a cohort of children with JSLE with the intention of subsequent longitudinal follow-up. Despite a plethora of studies examining CVD risk in adults with SLE the data describing CVD in JSLE is both sparse and conflicting, thus it is tempting to simply extrapolate from adult studies. Adult data suggests an increased risk of CVD in JSLE since, in adults, greater disease duration, increased disease severity and the more frequent prevalence renal disease is associated with an increased incidence of CV events. However children are not just little adults and despite having a more aggressive disease phenotype their vessels may well display an adaptive phenotype.

In addition, many of the adult studies have been marred by the prevalence of traditional CV risk factors amongst adult patients, such as smoking and a lifetime of obesity, which should not be present among children. Thus, it was hoped that characterising the vascular phenotype of children with JSLE would add valuable information to the adult SLE literature. In the previous chapter I have outlined the low prevalence of traditional CV risk factors, such as obesity, inactivity and smoking, amongst this cohort and the increased burden of SLE-related risk factors, such as renal impairment, disease activity and hypertension.

In this chapter I will test the hypotheses that JSLE is associated with an abnormal vascular phenotype and that it is associated with the the presence of hypertension, renal impairment, the use of corticosteroids and the duration of disease.

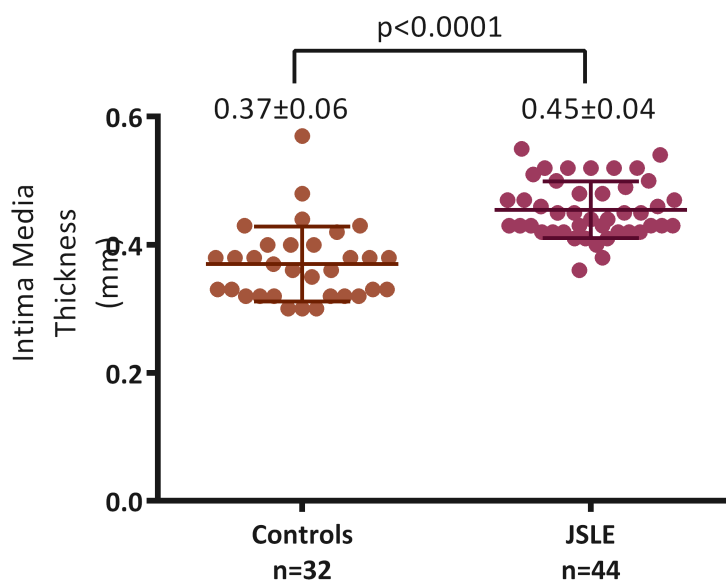
## Results

45 children and young adults with JSLE were recruited to the study. Of these 44 consented to have all vascular scans performed, and one patient was unable to remain sufficiently still for the PWV scanning. 35 were also recruited to the UK JSLE Cohort Study and 29 had both BILAG scores calculated and vascular scans performed. Comparison with control data generated by Dr Rukshana Shroff in the Vascular Physiology Department in Great Ormond Street has been used with her generous permission.(62)

### Carotid Intima Media Thickness

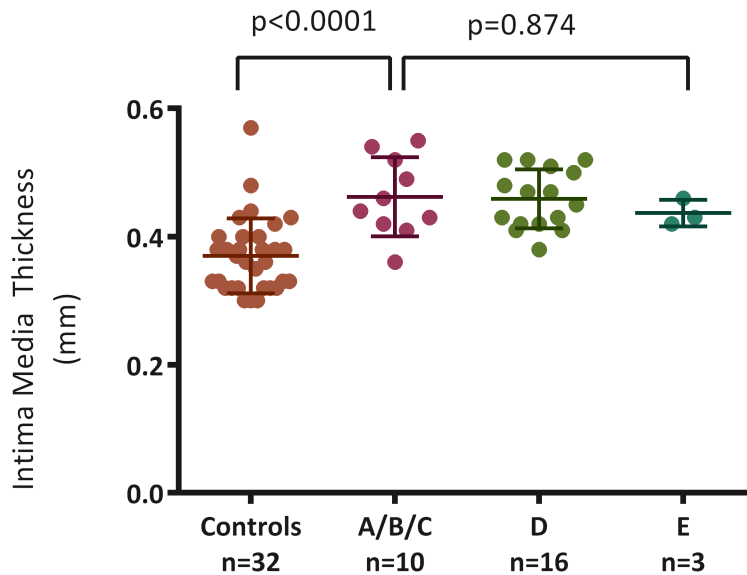
44 children and young adults with JSLE had cIMT measured and the control group consisted of 32 patients. Mean cIMT for the control group was  $0.37\text{mm} \pm 0.06$  and for the JSLE group was  $0.45 \pm 0.04$ , see figure 17. Children and young adults with JSLE had a 23% increase in mean cIMT ( $p < 0.0001$ ).

**Figure 17. Comparison of carotid intima media thickness between controls and patients with juvenile-onset systemic lupus erythematosus**



There was no statistically significant association between cIMT and upper or lower quartiles of age, METs, BILAG score, BILAG renal score or disease duration, figure 18. An increase in cIMT was also not associated with male gender or a family history of CVD. However, cIMT did correlate weakly with sitting minutes/week ( $r^2 = 0.234$ ,  $p=0.176$ ) and BMI centile ( $r^2 = 0.276$ ,  $p=0.077$ ). See also tables 19 – 21. For upper and lower quartile set points for cIMT and PWV see table 22. Those with a serum cholesterol greater than 5mmol/L ( $n=9$ ) had a cIMT of  $0.47 \pm 0.05$ mm whereas those with a serum cholesterol below 5mmol/L ( $n=36$ ) had a cIMT of  $0.45 \pm 0.04$ mm,  $p=0.0271$ .

**Figure 18. Comparison of carotid intima media thickness between controls and patients with juvenile-onset systemic lupus erythematosus by renal pBILAG-2004 score**



**Table 19. Demographic, clinical and anthropometric characteristics of children and young adults with juvenile-onset systemic lupus erythematosus**

	<b>Children with JSLE (n=45)</b>
<b>Age (months)</b>	162±35
<b>Gender (♀:♂)</b>	39:6
<b>Weight (kg)</b>	50.5(36.5-62)
<b>Height (cm)</b>	150.1±16
<b>BMI</b>	21.63(18.19-24.57)
<b>BMI centile</b>	65.63±28.8
<b>Disease duration (months)</b>	39(20.5-64.5)
<b>Age at diagnosis (months)</b>	115 ±42
<b>Cigarette smoking</b>	0
<b>Oral contraceptive pill</b>	1
<b>Positive family history of CVD</b>	11
<b>Current Prednisolone mg/kg</b>	0.07(0-0.23)
<b>METS/week</b>	1773(676.5-2854)
<b>Sitting minutes/day</b>	505±144.2
<b>Mean cIMT (mm)</b>	0.445(0.42-0.49)
<b>Mean PWV (m/s)</b>	5.272 ±0.88

All figures mean ± standard deviation or median (inter-quartile range)

**Table 20. Correlation between clinical findings, laboratory markers and vascular phenotype**

	Mean cIMT		PWV	
	r <sup>2</sup>	p	r <sup>2</sup>	p
Age at Diagnosis				
Disease Duration			0.374	0.019
Age			0.345	0.023
SBP			0.352	0.022
METS/week				
Sitting Minutes/week	0.234	0.176		
VLDL				
LDL			0.371	0.048
BMI centile	0.276	0.077		
eGFR			0.331	0.055

**Table 21. PWV and cIMT by BILAG score**

BILAG Renal Score	n	Mean cIMT	Mean PWV
A/B	5	0.45±0.07mm	5.7±1.09m/s
C	5	0.48±0.04mm	5.18±0.88 m/s
D	14	0.46±0.05mm	5.24±0.79 m/s
E	3	0.44±0.02mm	5.43±0.95m/s

All figures mean ± standard deviation

**Table 22. Upper and lower quartile set points for carotid intima media thickness (cIMT), pulse wave velocity in patients with juvenile-onset systemic lupus erythematosus**

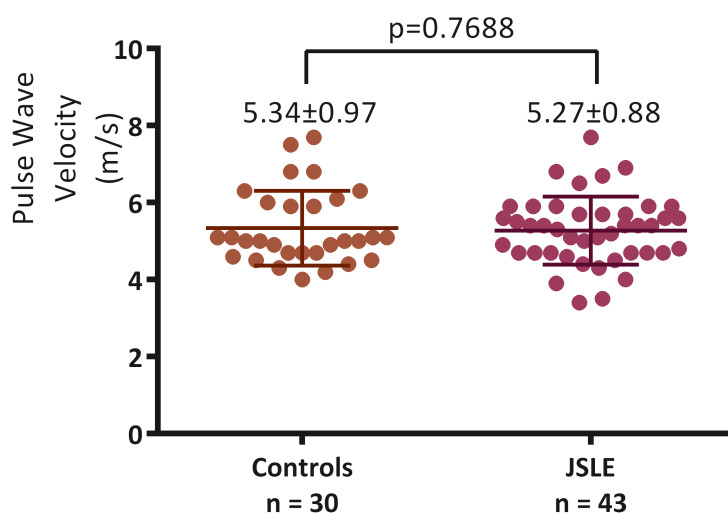
	N	Mean	Median	Minimum	Maximum	Lower Quartile	Upper Quartile
<b>cIMT</b>	44	0.4543	0.4450	0.36	0.55	0.42	0.4875
<b>PWV</b>	43	5.272	5.4	3.4	7.7	4.7	5.7

### Pulse Wave Velocity

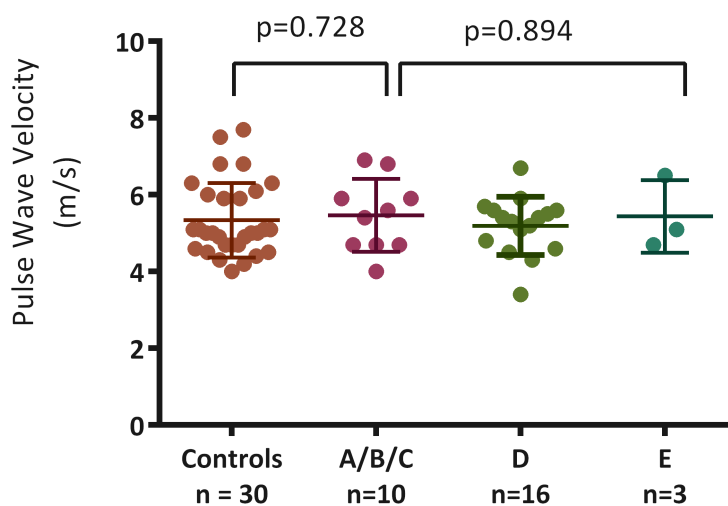
43 children and young adults with JSLE had PWV measured and the control group consisted of 30 children and young adults. Mean PWV for the control group was 5.34m/s  $\pm$ 0.97, see figure 19. Mean PWV for the patient group was 5.27m/s  $\pm$ 0.88. Children and young adults with JSLE had a 1.2% decrease in PWV and this was not statistically significant (p=0.77). Increased PWV was associated with disease duration ( $r^2$  =0.374, p=0.019), age ( $r^2$  =0.345, p=0.023), systolic blood pressure (SBP) ( $r^2$  =0.352, p=0.022), LDL ( $r^2$  =0.371, p=0.048) and eGFR ( $r^2$  =0.331, p=0.055).

There was no statistically significant association between PWV and upper or lower quartiles of age, BILAG score, BILAG renal score, prednisolone dose, METs or disease duration, see figures 20 and 21. An increase in PWV was also not associated with male gender or a family history of CVD, see tables 19 – 21. Those with a serum cholesterol greater than 5mmol/L (n=9) had a PWV of 5.49 $\pm$ 0.7m/s whereas those with a serum cholesterol less than 5mmol/L (n=36) had a tendency to a lower PWV of 5.24m/s, although this did not reach statistical significance, p=0.433.

**Figure 19. Comparison of pulse wave velocity between controls and patients with juvenile-onset systemic lupus erythematosus**

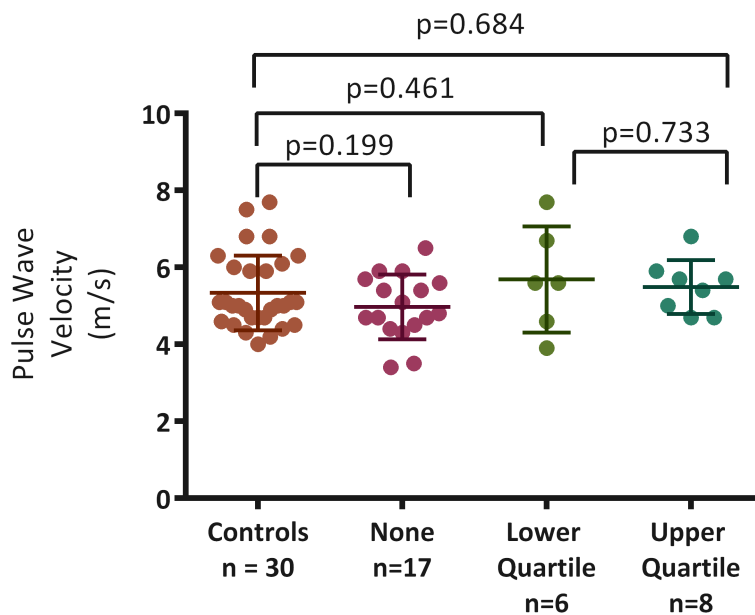


**Figure 20. Comparison of pulse wave velocity between controls and patients with juvenile-onset systemic lupus erythematosus by renal pBILAG-2004 score**





**Figure 21. Comparison of pulse wave velocity between controls and patients with juvenile-onset systemic lupus erythematosus by daily dose of prednisolone divided in to none, lower and higher quartiles in mg/kg/day**



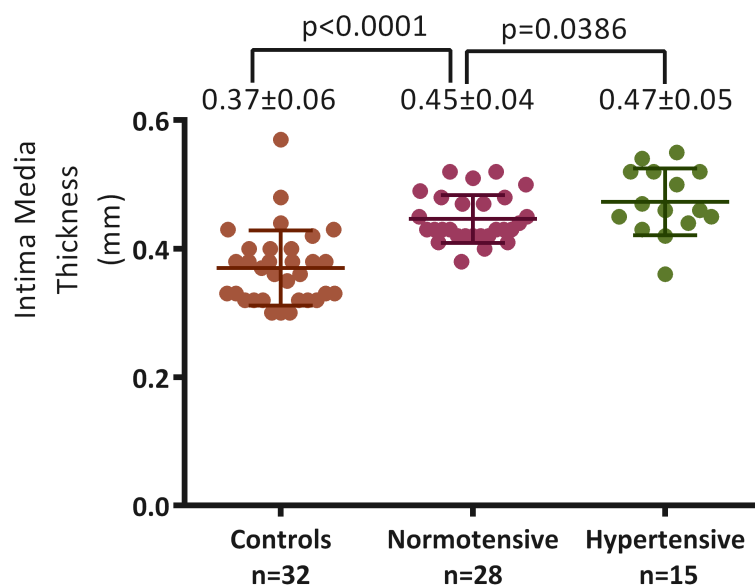
### Hypertension and the Vascular Phenotype

There was a significant association between the use of anti-hypertensives and an increase in cIMT. In the normotensive group not treated with anti-hypertensives cIMT was  $0.45 \pm 0.04$  mm and in the group treated with anti-hypertensives it was  $0.47 \pm 0.05$  mm,  $p=0.04$ . The relationship between anti-hypertensive use and cIMT remained significant even after controlling for total BILAG score, prednisolone dose, age, family history of CV disease and gender, ( $p=0.04$ ). In the absence of anti-hypertensive medication there was a higher cIMT of 21% from controls,  $p<0.0001$ . See figure 22.

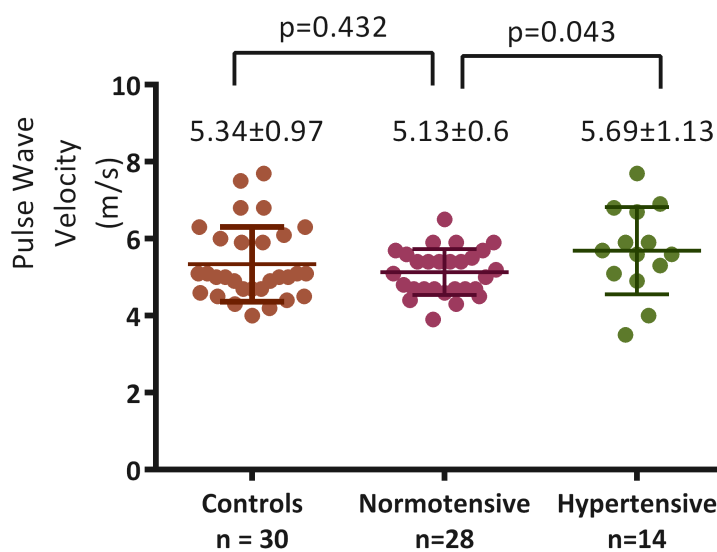
There was a significant association between the use of anti-hypertensives and an increase in PWV. In the normotensive group, not treated with anti-hypertensives, PWV was  $5.13 \pm 0.6$  m/s and in the group treated with anti-hypertensives it was  $5.69 \pm 1.13$  m/s,  $p=0.043$ . Similar to the cIMT findings, PWV was increased from controls, even in the absence of anti-hypertensives from  $5.34 \pm 0.97$  to  $5.13 \pm 0.06$ , however this did not meet statistical significance,  $p=0.132$ . See figure 23

As stated previously, due to small numbers, HTN was defined as both elevated BP and the use of anti-hypertensive medications. However, since the commonest anti-hypertensive in use was an ACE-inhibitor, it is likely that many of those defined as hypertensive may have had only low level HTN and proteinuria.

**Figure 22. Comparison of carotid intima media thickness between controls and patients with juvenile-onset systemic lupus erythematosus by use of anti-hypertensive medication**



**Figure 23. Comparison of pulse wave velocity between controls and patients with juvenile-onset systemic lupus erythematosus by use of anti-hypertensive medication**

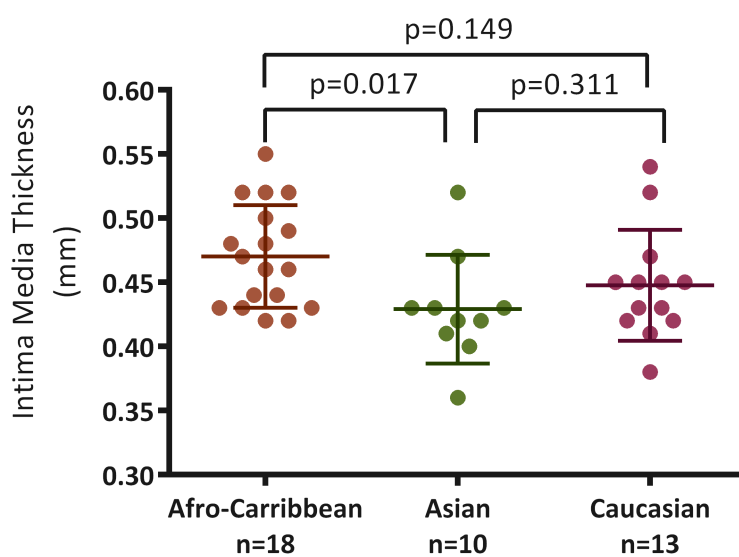


## Ethnicity and the Vascular Phenotype

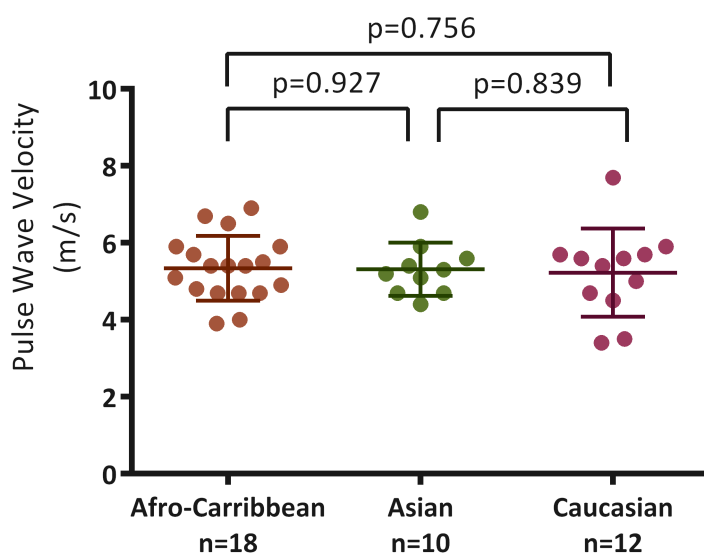
When analysed by ethnicity there was a significant difference between the Asian population and both the Caucasian and Afro-Caribbean population. As stated in the previous chapter, 19 children identified themselves as Afro Caribbean, 13 identified themselves as Caucasian and 10 identified themselves as Asian. 1 child was Afghani, 1 was Turkish Cypriot and 1 declined to answer this question. 1 Caucasian child was unable to remain still for the PWV reading and 1 Afro-Caribbean child declined consent for vascular scanning. Mean cIMT was highest in the Afro-Caribbean cohort at  $0.47 \pm 0.04$ mm lowest in the Asian cohort at  $0.429 \pm 0.04$ mm. In the Caucasian cohort it was  $0.448 \pm 0.04$ mm. This difference was statistically significant for the Afro-Caribbean cohort compared to the Asian cohort,  $p=0.017$ , but did not meet statistical significance when comparing the other cohorts. See figure 24.

There was a similar difference noted among the PWV results. Mean PWV was highest for the Afro-Caribbean cohort at  $5.339 \pm 0.74$ m/s but lowest for the Caucasian population  $5.225 \pm 1.148$ . The high standard deviation suggests greater spread amongst this group. Mean PWV among the Asian cohort was  $5.31 \pm 0.69$ m/s. None of the comparisons reached statistical significance. See figure 25.

**Figure 24. Comparison of carotid intima media thickness between patients with juvenile-onset systemic lupus erythematosus by ethnic background**



**Figure 25. Comparison of pulse wave velocity between patients with juvenile-onset systemic lupus erythematosus by ethnic background**



## Renal Function and the Vascular Phenotype

With regards to their vascular phenotype children with a grossly elevated UaUc (described previously) had an elevated cIMT  $0.455 (0.43 - 0.49)$  mm V  $0.435 (0.42 - 0.49)$  mm,  $p=0.89$  and a prolonged PWV  $5.564 \pm 0.72$  m/s V  $5.172 \pm 0.92$  m/s,  $p=0.2$ . However, the numbers were too small to reach statistical significance. See table 23.

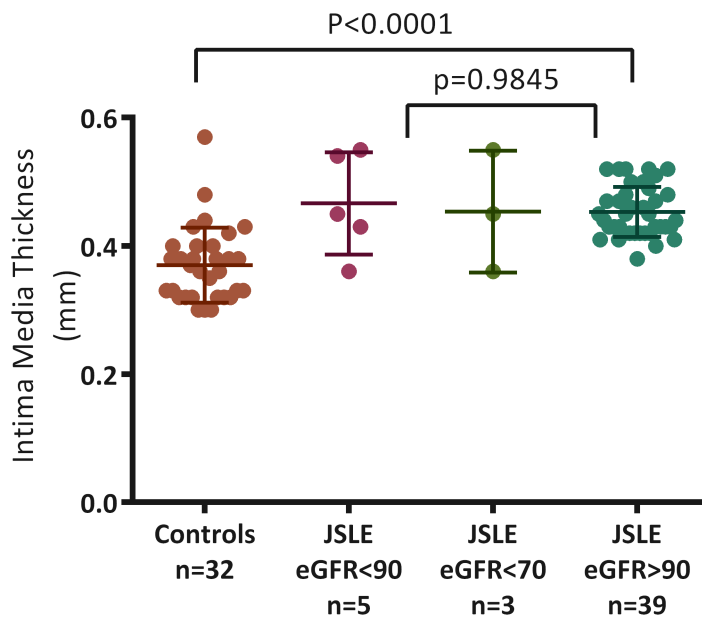
Looking at the small number of children with an abnormal eGFR showed that a lower eGFR was associated with an abnormal PWV but not cIMT. Median cIMT for the children with an eGFR  $<90$  ml/min/1.73m<sup>2</sup> was  $0.45 (0.4 - 0.5)$  mm and for those with an eGFR  $<70$  ml/min/1.73m<sup>2</sup> was  $0.45 (0.36 - 0.55)$  mm. There was no difference when compared to children with JSLE and an eGFR  $>90$  ml/min/1.73m<sup>2</sup>. The difference between medians was not significant ( $p=0.556$ ). However, median PWV for children with an eGFR  $<90$  ml/min/1.73m<sup>2</sup> was  $5.7 (5.5 - 6.6)$  m/s and for those with an eGFR  $<70$  ml/min/1.73m<sup>2</sup> was  $6.8 (5.7 - 6.9)$  m/s. This is an increase of  $1.1$  mm when compared to children with an eGFR  $>90$  ml/min/1.73m<sup>2</sup> and this reached statistical significance despite the small numbers,  $p=0.0277$ . See figures 26 and 27.

24 of the 45 children had LN proven on renal biopsy. Those who did not have a biopsy and those who had a biopsy that did not show LN were groups together for analysis, since those who had not been biopsied had not a clinical suspicion of LN. cIMT of those with biopsy proven LN was  $0.45 (0.43 - 0.49)$  mm and for those who had either not had a biopsy, or had a biopsy without LN cIMT was  $0.43 (0.42 - 0.49)$  mm,  $p = 0.619$ . PWV of those with biopsy proven LN was  $5.35 (4.75 - 5.9)$  m/s and those without was  $5.4 (4.6 - 5.65)$  m/s,  $p=0.138$ .

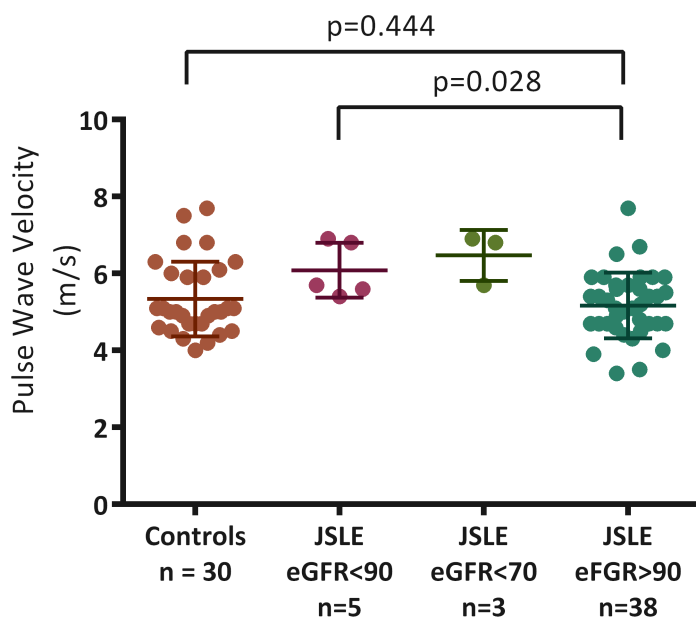
Comparing those with active versus quiescent or no LN was not significant. Those with a renal BILAG score of A/B/C had a cIMT of  $0.46 \pm 0.06$  mm whereas those with a score of D/E had a cIMT of

0.45±0.04mm, p=0.73. Those with a renal BILAG score of A/B/C had a PWV of 5.46±0.95m/s whereas those with a score of D/E had a PWV of 5.226±0.76, p=0.78.

**Figure 26. Comparison of carotid intima media thickness between controls and patients with juvenile-onset systemic lupus erythematosus by estimated glomerular filtration rate (eGFR) divided into >90, <90 and <70 ml/min/1.73m<sup>2</sup>**



**Figure 27. Comparison of pulse wave velocity between controls and patients with juvenile-onset systemic lupus erythematosus by estimated glomerular filtration rate (eGFR) divided into >90, <90 and <70 ml/min/1.73m<sup>2</sup>**





**Table 23. Demographic, clinical and anthropometric characteristics of children and young adults with JSLE, normal and grossly elevated UaUc**

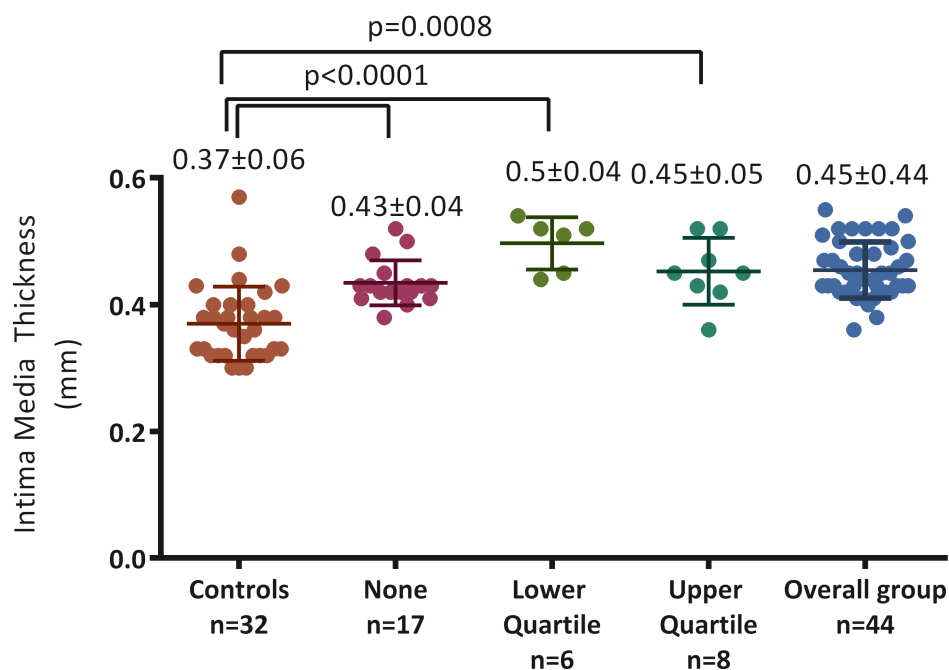
	Children with JSLE (n=45)	UaUc <20mg/mmol (n=33)	UaUc >20mg/mmol (n=12)
<b>UaUc (mg/mmol)</b>	2.9(1.2-55.3)	1.7(0.95-3.5)	164.6(55.3-304.9)
<b>Age (months)</b>	162±35	160±38	165±20
<b>Gender (♀:♂)</b>	39:6	29:4	10:2
<b>BMI</b>	21.6(18.2-24.6)	21.3(18.2-24.3)	22.29(18.7-25.2)
<b>BMI centile</b>	65.63±28.8	63.7±29	70.5±28.8
<b>Disease duration (months)</b>	39(20.5-64.5)	22(21.8-71)	36.5(7.5-49.5)
<b>Positive family history of CVD</b>	11 (24.4%)	9 (27.3%)	2 (16.6%)
<b>Treatment for hypertension</b>	16 (35.5%)	9 (27.3%)	8 (66.7%)
<b>METS/week</b>	1773(676.5-2854)	2190(1127-3306)	886(615-1648)
<b>Median cIMT (mm)</b>	0.445(0.42-0.49)	0.435(0.42-0.495)	0.455(0.43-0.49)
<b>Mean PWV (m/s)</b>	5.272±0.88	5.172±0.92	5.564±0.72

*All figures mean ± standard deviation or median (inter-quartile range)*

## Medications and the Vascular Phenotype

There were significant increases in cIMT measurements between controls and patients receiving no prednisolone, and those receiving the lower and upper quartiles of prednisolone (0.087 and 0.315mg/kg respectively). The cIMT of those receiving no prednisolone measured  $0.43\pm0.04\text{mm}$ , and this increased to  $0.5\pm0.04\text{mm}$  for those on low doses, and  $0.45\pm0.05\text{mm}$  for those on higher doses. There was a significant difference between controls and those on all doses of prednisolone ( $p<0.05$ ) and between those on no prednisolone and the lower quartile ( $p=0.0018$ ), see figure 28.

**Figure 28. Comparison of carotid intima media thickness between controls and patients with juvenile-onset systemic lupus erythematosus by daily dose of prednisolone divided in to none, lower and higher quartiles in mg/kg/day**



### Traditional Cardiovascular Risk Factors and the Vascular Phenotype

10 children had a positive family history of CVD as defined by Expert Panel on Integrated Guidelines for Cardiovascular health and Risk Reduction and described in the methodology chapter. These children had a higher PWV than those with a negative family history of CVD, 5.6 (4.65 – 6.25)m/s V 5.25 (4.7 – 5.75)m/s, but this did not reach statistical significance. However, this was not reflected in their cIMT which was marginally lower in the group with a positive family history, 0.43 (0.4 – 0.48)mm than in those with a negative family history, 0.45 (0.43 – 0.49)mm.

## Discussion

This cohort has shown an increase in cIMT, a structural change in the vessel wall, but interestingly has not shown a significant increase in PWV, a functional measurement of vessel health. Indeed, the median PWV was lower in the JSLE cohort than in controls, suggesting alternate pathways for the development of CVD in JSLE or a degree of compensation in the early stages of SLE, which may be overwhelmed in time. The APPLE study showed a mean mean cIMT progression over 36 months of 0.0017mm in pre-pubertal children and 0.0029mm in post pubertal children whereas the Whitehall study (350) showed a progression in cIMT of  $0.012 \pm 0.028$ mm/year in healthy adults aged 45-65 years.

Other groups using the same protocol have shown an increase in PWV with greater disease activity without a change in cIMT. The AddIT study (315), which used the same protocols as ours, showed a cIMT of  $0.44 \pm 0.04$ mm and a PWV of  $4.86 \pm 0.7$ m/s in adolescents with well-controlled diabetes and a cIMT of  $0.44 \pm 0.05$ mm and PWV of  $5.0 \pm 0.84$ m/s in those with poor control and proteinuria.

The increase in both cIMT in our cohort is most marked in the hypertensive patients, though even the normotensive patients had increased measurements when compared to the control group, who were all normotensive. All of these patients were maintained on anti-hypertensives, largely ACEIs, at the time of the study, thus the difference between groups may be explained by renal involvement or severity of disease. However, there was no correlation found between cIMT or PWV and either the overall or the renal BILAG score, or indeed the prednisolone dose, which could be presumed to be a reasonable surrogate marker for disease severity.

This study has shown no difference in BMI centiles between patients and controls, a low prevalence of sedentary behavior and no smoking. Indeed in contrast to their adult counterparts(89) this cohort of children with JSLE had high levels of activity with a median of 1773 METs per week, corresponding to a high level of physical activity. Encouraging an active lifestyle during clinical encounters is one way in which paediatricians could potentially reduce their patients' burden of CV risk in the future. The data should be interpreted with caution, however, since this is a small cohort and a cross-sectional study of children in different phases of their disease.

In summary, while this data does not offer conclusive evidence of an increased risk of CVD in UK-based patients with JSLE, it is suggestive of early structural changes, which may become clinically significant with time. There has also been significant heterogeneity in methodology and a lack of controls cohorts in much of the published paediatric data but the evidence suggests that patients with JSLE may have an increased burden of CVD. Follow-up data from this cohort is needed to determine if the early compensatory changes resulting in normal PWV readings are overwhelmed with time and if the increase in pre-clinical markers of CVD are followed by hard CV endpoints. In the next chapter I will examine the role of adipokines in an altered vascular phenotype in JSLE.



# **Adipokine Dysfunction in Children with Juvenile-onset Systemic Lupus Erythematosus**

## Introduction

Obesity is a well-established traditional CV risk factor and is common in adults with SLE. Depending on the method used to define obesity (ie body mass index (BMI)  $>30\text{kg/m}^2$ , waist circumference  $>88\text{cm}$  or waist:hip ratio  $>0.85$ ) it affects up to 50% of adults with SLE.(87) Increased waist circumference and obesity have been independently associated with increased cIMT and PWV in adult individuals with SLE(45,74,88).

As outlined previously there has been a shift in thinking about adipose tissue in the last decade – rather than being considered merely a passive storage place for energy it is now recognised as an active endocrine organ. Among other cytokines it secretes leptin and adiponectin, which interact with the immune system and the vasculature to predispose the individual to CVD. As mentioned previously, adult studies have shown SLE to be a hyperleptinaemic and hypoadiponectinaemic condition. I will not reiterate the potential modes of action of these cytokines here, as this has been extensively covered previously.

In this chapter I will explore the hypothesis that the increased risk of CVD in JSLE is related to increased disease activity leading to abnormalities in adipokine activity.



## Results

### Demographics

25 children and young adults with JSLE were recruited to this section of the study, of these 24 consented to have all vascular scans performed, and one patient was unable to remain sufficiently still for the PWV scanning. The mean age of the children was  $123 \pm 43$  months at diagnosis and  $170 \pm 27.7$  months at recruitment, 22 were female. 15 children and young adults were recruited as a control group. The mean age was  $114.1 \pm 18.4$  months, 7 were female. See table 24.

**Table 24. Demographic, clinical, anthropometric and biochemical characteristics of children and young adults with SLE**

	<b>Children with JSLE (N=25)</b>	<b>Controls (N=15)</b>
<b>Age (months)</b>	170 ±27.7	114±18.4
<b>Gender (♀:♂)</b>	22:3	8:7
<b>BMI</b>	22.12±4.26	21.52±4.45
<b>BMI Centile</b>	67.55±26.4	81.75±24.97
<b>eGFR (ml/min/1.73m<sup>2</sup>)</b>	99.6 (89.9-113.9)	
<b>Disease duration (months)</b>	33 (20.8-67.3)	
<b>Age at diagnosis (months)</b>	123 ±43	
<b>Cigarette smoking</b>	0	
<b>Oral contraceptive pill</b>	1	
<b>Positive family history of CVD</b>	8	
<b>METS</b>	1499 (526-3087)	
<b>Sitting minutes/day</b>	540 (480-840)	
<b>Mean cIMT (mm)</b>	0.4588 ±0.04875	
<b>Mean PWV (m/s)</b>	5.287 ±0.727	
<b>hsCRP</b>	0.25 (0.1-0.67)	
<b>Cholesterol (mmol/l)</b>	5.262 ±1.476	
<b>Triglycerides (mmol/l)</b>	1.495 ±1.082	
<b>HDL (mmol/l)</b>	1.4 ±0.476	
<b>VLDL (mmol/l)</b>	0.6478 ±0.455	
<b>LDL (mmol/l)</b>	3.18 ±1.34	
<b>ESR</b>	16 (6.7-44.25)	
<b>UaUc</b>	3.95 (1.35-111.8)	
<b>Ferritin</b>	31 (18-72)	

All figures mean ± standard deviation or median (inter-quartile range)

## Cardiovascular Risk Factors

All children with JSLE denied smoking, 1 had recently been commenced on the oral contraceptive pill and 8 had a positive family history of cardiovascular disease. Measures of physical activity varied greatly with a median of 1499 (526 – 3087) METs/week, corresponding to a moderate degree of activity. Daily sitting time also varied greatly with a median of 540 (480 – 840) minutes/day.

Serum cholesterol was  $5.262 \pm 1.476$  mmol/L, Triglycerides were  $1.495 \pm 1.082$  mmol/L, HDL was  $1.495 \pm 1.082$  mmol/L, VLDL was  $0.6478 \pm 0.455$ , LDL was  $3.18 \pm 1.34$  mmol/L. GFR was estimated at  $95.36 \pm 29$  ml/min/1.73m<sup>2</sup> and 4 children had an eGFR <90 ml/min/1.73m<sup>2</sup>. Median BMI centile for the control group was 88.6 (24 – 98) and for the JSLE group was 76.8 (36.8 – 88.6) and the difference between groups was significant,  $p=0.044$ .

## Leptin

Leptin levels were 16.52(8.27 – 27.27)ng/ml in the JSLE group and 7.56(0.99-16.7)ng/ml in the control group, the difference between the medians was significant ( $p=0.0238$ ). See figure 29. Significant correlations were found between leptin levels and systolic BP ( $r^2=0.482$ ,  $p=0.0172$ ), PWV ( $r^2=0.433$ ,  $p=0.039$ ), serum LDL ( $r^2=0.585$ ,  $p=0.0137$ ) and BMI centiles ( $r^2=0.540$ ,  $p=0.0078$ ) in the JSLE group. See table 25. Comparing leptin levels by renal BILAG score showed a tendency towards increased levels in those with active renal disease (A/B/C) but this did not reach statistical significance, see figure 30. However, those with an elevated UaUc (>20mg/mmol) had a corresponding rise in their serum leptin levels,  $35.5 \pm 38.7$  ng/ml when compared to those with low to normal UaUc (<20mg/mmol)  $18.79 \pm 13.3$  ng/ml. See table 26 and figure 31. Serum leptin in those with a history of biopsy proven LN was 17.56 (5.51 – 50) ng/ml whereas those with a history of a biopsy without

LN, or no biopsy, had a serum leptin of 15.15 (9.27 – 26.45) ng/ml, p=0.271.

**Table 25. Correlations between clinical factors and adipokine levels in patients with JSLE**

	Leptin		Adiponectin	
	r <sup>2</sup>	p	r <sup>2</sup>	p
<b>Age at Diagnosis</b>	0.345	0.0987	-0.159	0.4573
<b>Age(M)</b>	0.352	0.0847	<b>-0.416</b>	<b>0.0387</b>
<b>cIMT</b>	0.196	0.36	<b>0.433</b>	<b>0.039</b>
<b>PWV</b>	-0.232	0.27	0.225	0.302
<b>Systolic BP</b>	<b>0.482</b>	<b>0.0172</b>	0.002	0.9940
<b>Sitting Time</b>	0.248	0.2321	<b>0.440</b>	<b>0.0276</b>
<b>HDL</b>	0.045	0.8638	0.652	0.0046
<b>LDL</b>	<b>0.585</b>	<b>0.0137</b>	0.267	0.3007
<b>BMI centile</b>	<b>0.540</b>	<b>0.0078</b>	-0.220	0.3130
<b>eGFR</b>	-0.333	0.1297	-0.210	0.3473

**Table 26. Serum adipokine levels in children with JSLE analysed by urine albumin to creatinine ratio (UaUc)**

	UaUc <20mg/mmol (n=17)	UaUc >20mg/mmol (n=8)
<b>Leptin</b>	18.79±13.3	35.5±38.7
<b>Adiponectin</b>	10.5±7.13	21.9±9.5

All figures mean ± standard deviation

Figure 29. Comparison of serum leptin levels between controls and patients with juvenile-onset systemic lupus erythematosus

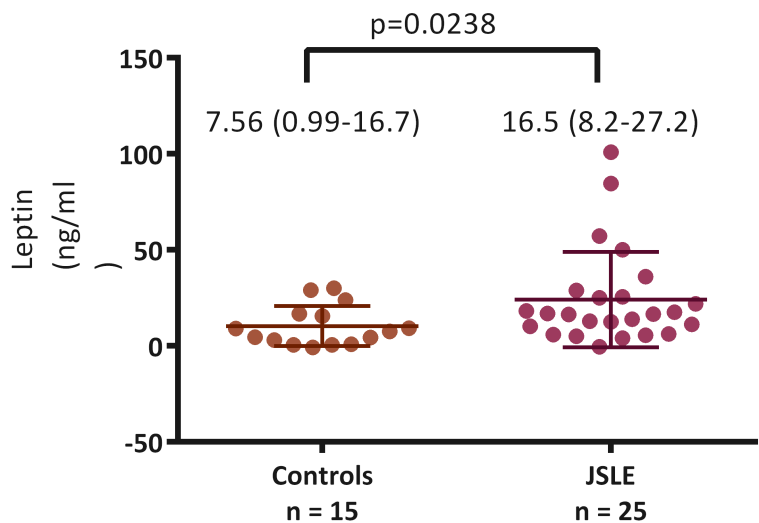
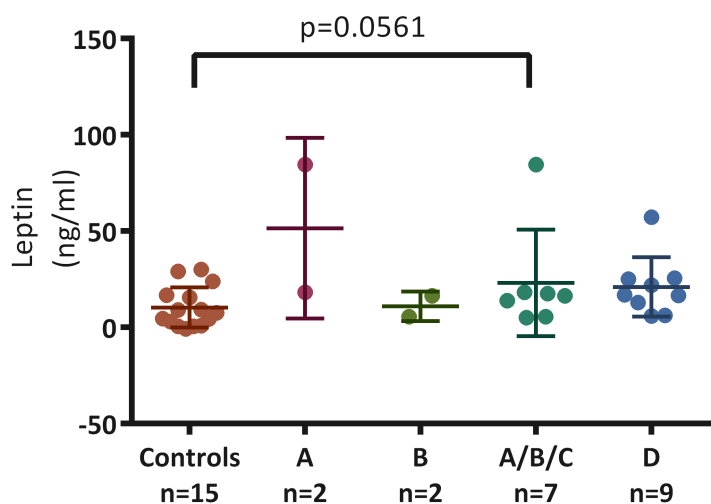
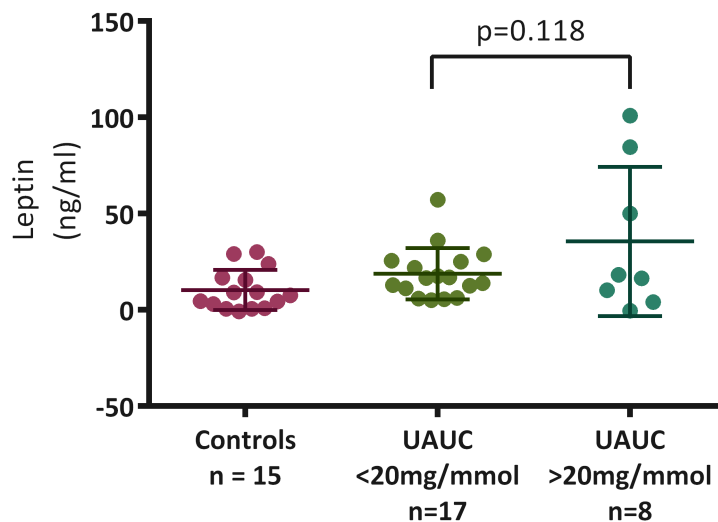


Figure 30. Comparison of serum leptin levels between controls and patients with juvenile-onset systemic lupus erythematosus by renal pBILAG-2004 score

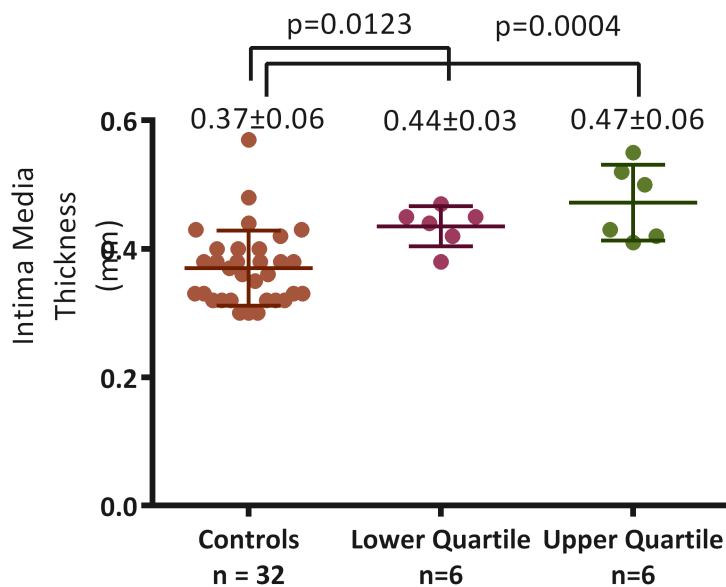


**Figure 31. Comparison of serum leptin levels between controls and patients with juvenile-onset systemic lupus erythematosus by urine albumin to creatinine ratio**

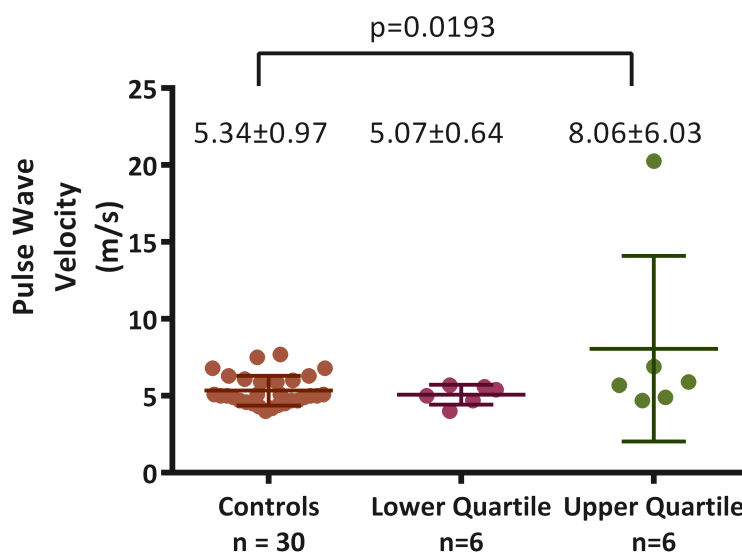


The cIMT increased with increasing leptin levels when analysed by quartiles. The lower leptin quartile group had a cIMT of  $0.44 \pm 0.03$ mm increasing to  $0.47 \pm 0.06$ mm in the higher quartile group,  $p=0.0004$ . See figure 32. There was a tendency to an increased PWV between upper and lower quartiles, but numbers were too small to reach statistical significance. See figure 33. See table 27 for upper and lower quartile see points for leptin.

**Figure 32. Comparison of carotid intima media thickness in patients with juvenile-onset systemic lupus erythematosus by lower and higher quartiles of serum leptin**



**Figure 33. Comparison of pulse wave velocity in patients with juvenile-onset systemic lupus erythematosus by lower and higher quartiles of serum leptin**



**Table 27. Upper and lower quartile set points for leptin and adiponectin in patients with juvenile-onset systemic lupus erythematosus**

	N	Mean	Median	Min	Max	Lower Quartile	Upper Quartile
<b>Leptin</b>	25	24.14	16.52	0.48	100.9	8.274	27.27
<b>Adiponectin</b>	25	14.16	12.59	1.7	30.58	5.70	20.59

There was no difference in leptin levels depending on family history of CVD. Those with a positive family history of CVD had a median leptin level of 17.4 (7.8 – 27.9) ng/ml, while those with a negative family history CVD had a median leptin level of 16.4 (7.9 – 30.9) ng/ml.

#### **Adiponectin**

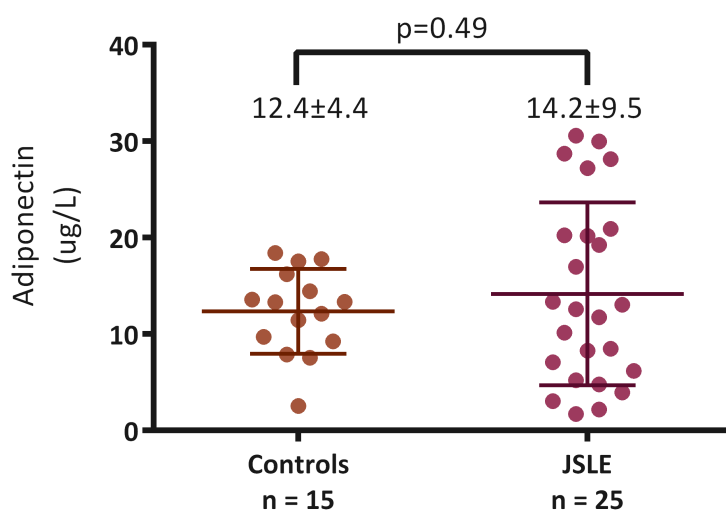
Adiponectin levels were  $14.2 \pm 9.5$  ug/ml in the JSLE group and  $12.4 \pm 4.4$  ug/ml in the control group. The difference between the means was not significant ( $p=0.49$ ) and there was no increase with renal BILAG score, see figures 34 and 35. Significant correlations were found between adipokine levels and age ( $r^2=0.416$ ,  $p=0.0387$ ) and weekly sitting time ( $r^2=0.440$ ,  $p=0.0276$ ). See table 25.

When divided into quartiles by adiponectin level, there was an increase in cIMT (from  $0.45 \pm 0.05$  to  $0.43 \pm 0.04$ ,  $p=0.39$ ) and PWV across the centiles ( $5.02 \pm 0.58$  to  $5.45 \pm 0.97$ ,  $p=0.37$ ), although this was not statistically significant for PWV, see figures 36 and 37.

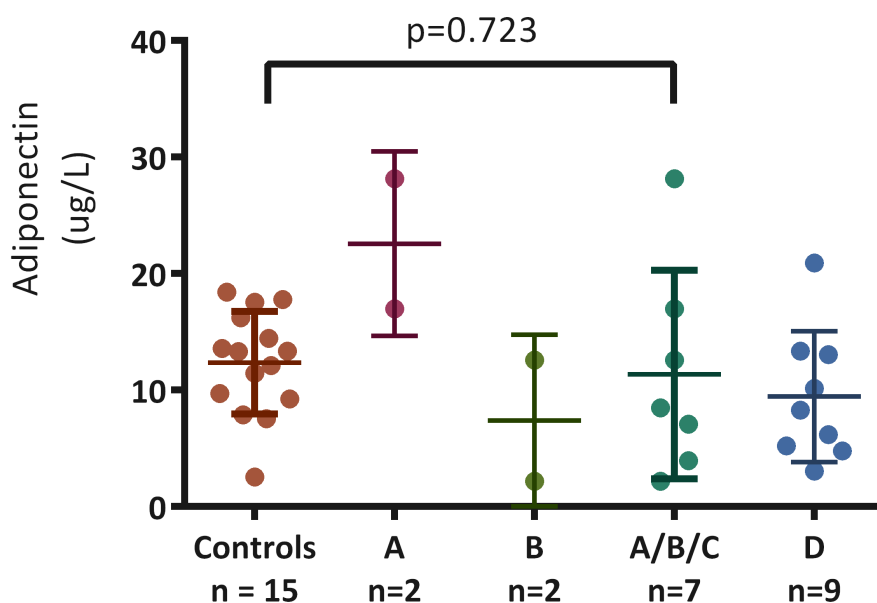
See table 27 for upper and lower quartile set points for adiponectin.



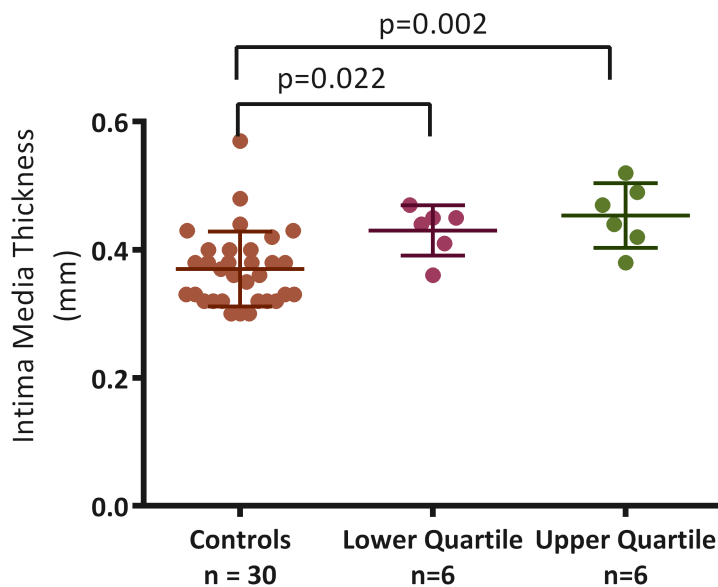
**Figure 34. Comparison of serum adiponectin levels between controls and patients with juvenile-onset systemic lupus erythematosus**



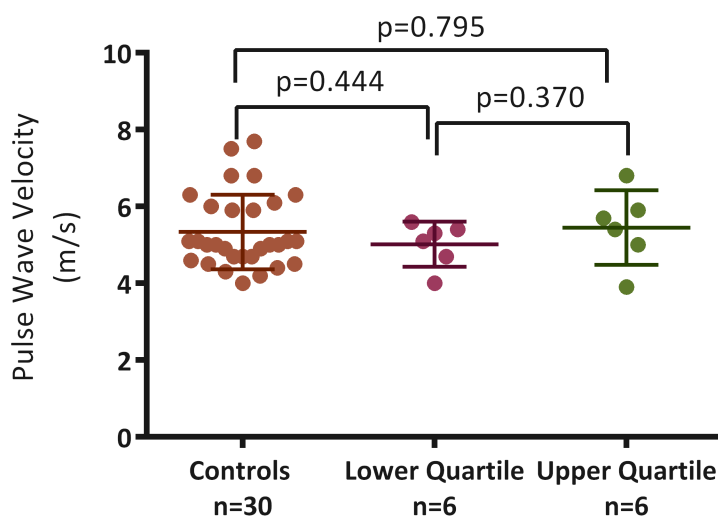
**Figure 35. Comparison of serum adiponectin levels between controls and patients with juvenile-onset systemic lupus erythematosus by renal pBILAG-2004 score**



**Figure 36. Comparison of carotid intima media thickness in patients with juvenile-onset systemic lupus erythematosus by lower and higher quartiles of serum adiponectin**



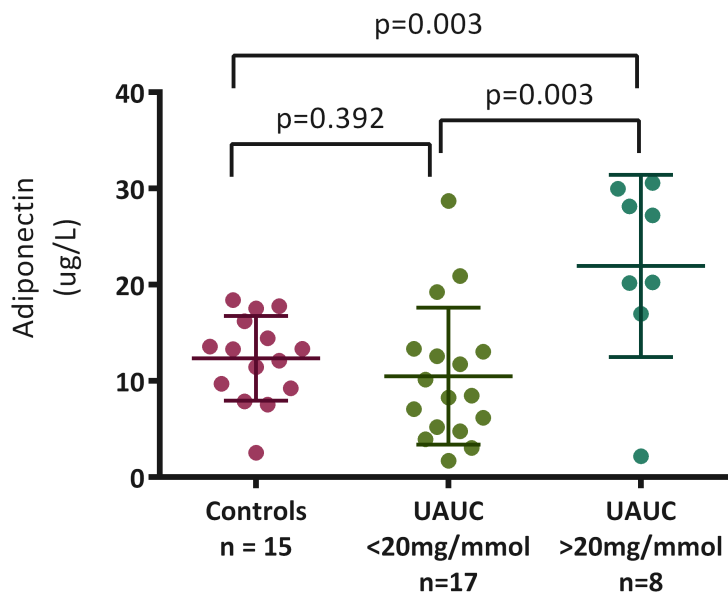
**Figure 37. Comparison of pulse wave velocity in patients with juvenile-onset systemic lupus erythematosus by lower and higher quartiles of serum adiponectin**



Similar to leptin, family history appeared to make no difference to adiponectin levels. Those with a positive family history of CVD had a median adiponectin level of 13.2 (7.6 – 20)ug/ml, while those with a negative family history CVD had a median adiponectin level of 10.15 (4.4 – 24)ug/ml.

Also similar to leptin, those with an elevated UaUc (>20mg/mmol) had a corresponding rise in their serum adiponectin levels,  $21.9 \pm 9.5$  ug/ml when compared to those with a low to normal UaUc (<20mg/mmol)  $10.5 \pm 7.13$  ug/ml. See table 26 and figure 38. Serum adiponectin in those with a history of biopsy proven LN was 19.25 (7.076 – 28.16) ug/ml whereas those with a history of a biopsy without LN, or no biopsy, had a serum adiponectin of 8.38 (2.86 – 12.15) ug /ml,  $p=0.0269$ .

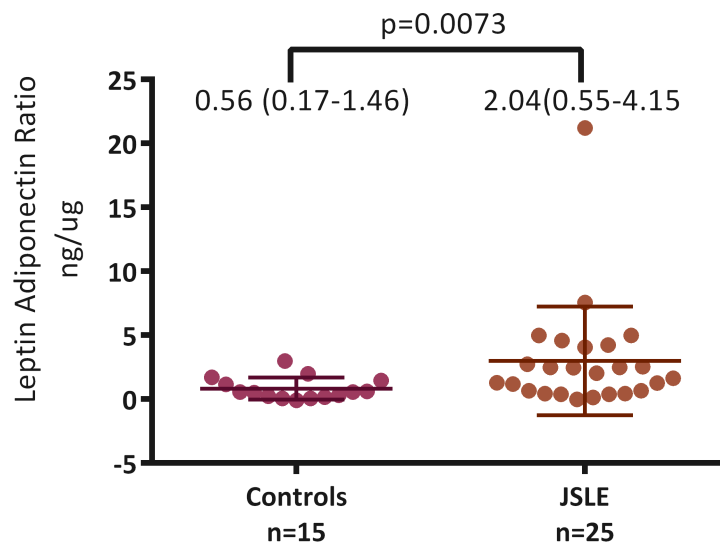
**Figure 38. Comparison of serum adiponectin levels between controls and patients with juvenile-onset systemic lupus erythematosus by urine albumin to creatinine ratio**



### Leptin Adiponectin Ratio

There was a significant increase in leptin adiponectin ratio in patients versus controls from 0.56 (0.17 – 1.46)ng/ug to 2.04 (0.55 – 4.15)ng/ug,  $p=0.0073$ . See figure 39.

**Figure 39. Comparison of leptin adiponectin ratio between controls and patients with juvenile-onset systemic lupus erythematosus**



### Discussion

SLE is associated with an increased prevalence of metabolic syndrome and patients have also been shown to have increased insulin resistance.(103) (104,105) Metabolic syndrome has been shown to be associated with an increased cardiovascular risk profile in patients with SLE and may contribute directly to accelerated atherosclerosis. (106) A combination of the reduction in physical activity secondary to fatigue or joint involvement, increased appetite due to glucocorticoid therapy and pre-existing obesity are all potential contributors to obesity in adult onset-SLE. The prevalence of obesity in JSLE has not been studied to

date, although this cohort have a median BMI above the 50<sup>th</sup> centile they fall below the control group in contrast to adult studies.(87,89)

Adults with SLE may have lower CV capacity, physical fitness, muscle strength and functional capacity than their peers(90) which results in reduced physical activity and exercise(97). This is potentially preventable however, with maintenance of adequate activity since arterial stiffening was not observed in habitually exercising adults with SLE in a study by Barnes et al (95). Low physical activity scores have been shown to be associated with pro-inflammatory HDL and increased subclinical atherosclerosis in women with SLE.(94) This has not been studied in the paediatric population.

In this cohort there was a striking difference in adipokine levels between the proteinuric patients and the non-proteinuric patients, with a doubling of both leptin and adiponectin levels patients with a UaUc > 20mg/mmol. Though these are small numbers they reflect what has been found in other populations. Proteinuria has been linked to raised serum leptin levels in chronic haemodialysis patients(340) but the study was not set up to determine whether the proteinuria and the raised leptin were both secondary to reduced renal function or whether the raised leptin caused the proteinuria. However, in examining pre-eclampsia, a poorly understood placenta mediated condition, specific to pregnancy, which results in proteinuria and hypertension, Ibrahim et al(341) investigated the effect of leptin administration on systolic blood pressure, proteinuria and serum markers of endothelial activation in the pregnant rat. They showed that leptin administration resulted in significantly increased urinary protein excretion, serum ICAM-1 levels and systolic blood pressure.

As outlined previously, in the literature review, adiponectin may have both anti-inflammatory effects and pro-inflammatory effects. In contrast to published data I found an increase in both leptin and adiponectin

between controls and patients. Given the role played by adiponectin in protecting endothelial nitric oxide mediated vasodilation I speculate that higher serum adiponectin levels early in the SLE phenotype could be an initial protective mechanism and could explain the increase in cIMT without an increase in PWV, However it could also be indicative of a pro-inflammatory adiponectin phenotype.

There is some clinical data to suggest that the leptin:adiponectin ratio could be predictive of atherosclerosis(342) based on surrogate outcome measures and this ratio was raised in JSLE patients when compared with controls. A recent European Study by Kappelle et al(343) showed that the Leptin:Adiponectin ratio was predictive of incident CVD, defined as death from CVD, hospitalisation for myocardial infarction, percutaneous transluminal coronary angioplasty or coronary artery bypass grafting in a group of 103 cases followed for a mean of 3 years when compared to 106 controls followed for a mean of 10 years. This link was lost with controlling for smoking, waist:hip ratio, microalbuminuria and serum lipids suggesting that the role of the leptin:adiponectin ratio is multifactorial.

Interestingly, there is some evidence to suggest that the protective effects of adiponectin and the damaging effects of leptin in potentiating atherosclerosis could disappear in individuals with advanced atherosclerotic disease(344,345) raising the possibility that in this cohort we are seeing an intermediate stage with high leptin and high adiponectin.

There are some limitations to this data. The control group had a different gender distribution with more males than the JSLE group, which may affect serum leptin levels. The ALSPAC group have shown higher leptin levels in obese females compared to males, though no difference in adiponectin levels. In this cohort however the gender breakdown was not significantly different for leptin (male controls 15.5(0.58-29)ng/ml V

female controls 6.1(3.45-20.3)ng/ml,  $p=0.93$ ) or for adiponectin (male controls 2.97(1.9-3.5)ng/ug V female controls 3.34(2.2-4.6)ng/ug,  $p=0.275$ ). A further limitation may be the lower BMI centiles for the JSLE patients, although this would be expected to be associated with lower leptin levels, rather than the higher levels found in this cohort.

In summary, our findings are in agreement with adult and paediatric studies and the relationship between serum adipokines and cIMT suggests that leptin could be used as a novel biomarker for CV risk in JSLE.





# **Conclusion: A Review of the Pathogenesis of CVD in JSLE**

## Introduction

The aim of this thesis was to examine CV risk in children with JSLE, to determine whether there is an increased risk of CVD and to analyse its epidemiology in an effort to understand its pathogenesis. I have shown that this cohort of children with JSLE have a structural change in their blood vessels, as shown by an increase in cIMT, without a corresponding functional change, demonstrated by a normal PWV. This structural change is particularly marked in children with renal inflammation, as measured by proteinuria, and in those of Afro-Caribbean background.

Furthermore I have shown an increase in both serum leptin and adiponectin in patients with JSLE, in contrast to adult data, and suggested that an increase in adiponectin could be a protective mechanism which would account for the preservation of PWV. It is possible that in time this protective mechanism will be overwhelmed and that follow-up investigations will show a deterioration in PWV.

## The Unique Pathogenesis of Cardiovascular Disease in Juvenile-onset Systemic Lupus Erythematosus

Drawing on the preceding literature review and the findings of this study I propose a pathogenesis of CVD in JSLE. The following steps are illustrated in figure 40, they are speculative and based on the previously quoted literature as well as the findings of this study.

1. The hallmark of SLE is the generation of auto-antibodies against healthy tissue, such as anti-dsDNA Abs, anti-vitamin D Abs, anti-HDL Abs, anti-cardiolipin Abs, and anti-C1q Abs. The damage caused by these antibodies results in tissue activation and a generalised state of systemic inflammation. Specific antibodies such as anti-HDL Abs reduce the body's defences against atherosclerosis by reducing reverse cholesterol transport. While within the vasculature a generalised state of inflammation results in results in increased endothelial inflammation,

increased endothelial cell permeability and increased endothelial adhesion of surface molecules. Further auto-antibodies, including anti-C1q Abs, lead to glomerulonephritis, kidney damage and proteinuria which further increases CVD (see point 6).

2. Increased serum levels of leptin are seen early in JSLE as evidenced by this cohort. This results in decreased endothelial cell nitric oxide mediated vasodilation reducing the blood vessels response to increased blood flow and increasing the damage caused to the endothelial cell by the sheer stress of the blood flow. Leptin also potentiates a pro-inflammatory Th1 response and decreased Treg function, resulting in an atherogenic phenotype. It stimulates CD4+ T cell proliferation and directly stimulates the production of leptin and INF- $\gamma$  by Th1 cells potentiating macrophage differentiation to foam cells. This differentiation is an initial protective step, designed to separate damaging ox-LDL molecules, but as the foam cells multiple to form a fatty streak they become an unstable atherosclerotic plaque.
3. In the early stages of JSLE, as seen in this cohort, increased serum levels of Adiponectin are seen. This initially acts in a pro-inflammatory fashion, similar to leptin, to potentiate a pro-inflammatory Th1 response, leading to increased adhesion of endothelial cell surface molecules and increased migration of macrophages. These macrophages initially act to phagocytose ox-LDL and wall it away in foam cells. Adult studies have shown low levels of adiponectin, later in the disease, leading to increase activation of the endothelium as outlined below.
4. The inflammatory milieu, leucocyte production of INF-alpha and the generation of antibodies against anti-inflammatory-HDL, leads to the generation of pro-inflammatory-HDL. This results in activation of the endothelium as outlined below and increased generation of oxidised LDL. A reduction in anti-inflammatory HDL leads to reduced reverse

cholesterol transport, increased generation of oxidised LDL and increased macrophage migration.

5. All of the above lead to endothelial and macrophage activation. Endothelial cell activation leads to increased cell permeability, increased adhesion of surface molecules which leads to increased migration of macrophages, decreased endothelial cell nitric oxide mediated vasodilation and increased endothelial cell apoptosis. Macrophage activation and migration into the sub-endothelial space acts to initially stabilise the inflammation by phagocytosis of oxidised LDL. This is further stimulated by production of INF- $\gamma$  by leptin activated, Th1 cells.
6. Renal inflammation, as evidenced by proteinuria and early chronic kidney disease, results in altered vitamin D activation. Combined with reduced serum levels of vitamin D, secondary to reduced sunlight and anti-vitamin D antibodies, this results in phenotypic transformation of the vascular smooth muscle cell. The vascular smooth muscle cells are stimulated by leptin to migrate and proliferate and migrate to stabilise the fledgling atherosclerotic plaque. Differentiation of the vascular smooth muscle cells now also leads to medial stiffening and thickening as the muscle cells undergo phenotypic transformation to become osteocytes, osteoblasts and chondrocytes.
7. Once formed, from foam cells, t-cells and vascular smooth muscle cells, the atherosclerotic plaque is self-potentiating. Narrowing of the vessel lumen leads to increased shear stress and further endothelial activation. As the plaque enlarges it inevitably ruptures, leading to the release of inflammatory mediators, platelet activation and clumping at the site of the rupture.

## Conclusion

In conclusion this cohort of children with JSLE show structural changes in their vessels indicative of early CVD but with adaptive changes resulting in normal functional scans.

The current evidence does not support pharmacological treatment of the increased CV risk in JSLE but encouraging children to keep active, maintain a healthy weight and avoid cigarettes is important as is the adequate treatment of their disease and any renal impairment. Educating children and their families about the increased CV risk in SLE is advisable with a particular emphasis on modification of traditional CV risk factors and the adoption of a healthy lifestyle. Somewhat discouraging though is that this cohort had a low prevalence of such modifiable factors and that, in adult practice, the adoption of a protocol that mandated regular assessment of CV risk factors in adult SLE clinics resulted in increased discussion of CVD but did not alter management. (292) Nonetheless encouraging our patients to maintain a healthy lifestyle remains an important part of preventive care.

Future research strategies should examine children more closely as they provide a model of aggressive SLE without the added CV risk factors or obesity, smoking or increased age. Longitudinal follow up of a large cohort provides the best opportunity to examine the evolution of CVD in JSLE and to examine the role played by aberrant adipokine activation, dysfunctional HDL, fetuin A, impaired macrophage function, abnormal endothelial activation, vitamin D and the plethora of idiosyncratic autoantibodies. Further studies should also focus on the contribution made by newer treatments such as rituximab and mycophenolate mofetil while examining the cumulative effects of more conventional treatments such as prednisolone, hydroxychloroquine and cyclophosphamide.

This study has many limitations. The cohort, though representative of the centre, does not represent the national cohort in terms of age and ethnicity, and is limited in size. Due to concerns about patient compliance and a lack of normative data for younger children, the vascular scans did not include FMD, which could have provided a more sensitive assessment of endothelial function. Serological investigation was limited by funding and if possible it would be interesting to assess, for example, HDL function in the future. Also the use of a retrospective control cohort, without ethnic matching, was not ideal and necessitated by failure to recruit healthy children within the hospital or siblings of patients. The use of common control cohorts within centres would be beneficial to all parties. Furthermore utilising current steroid dose rather than cumulative steroid load, though expedient, may have limited the findings. The development of CVD is a prolonged process, beginning in early childhood, and the cumulative steroid and disease load is arguably more significant than a one off reading which could reflect a short term change, whether positive or negative, in the individual's disease.

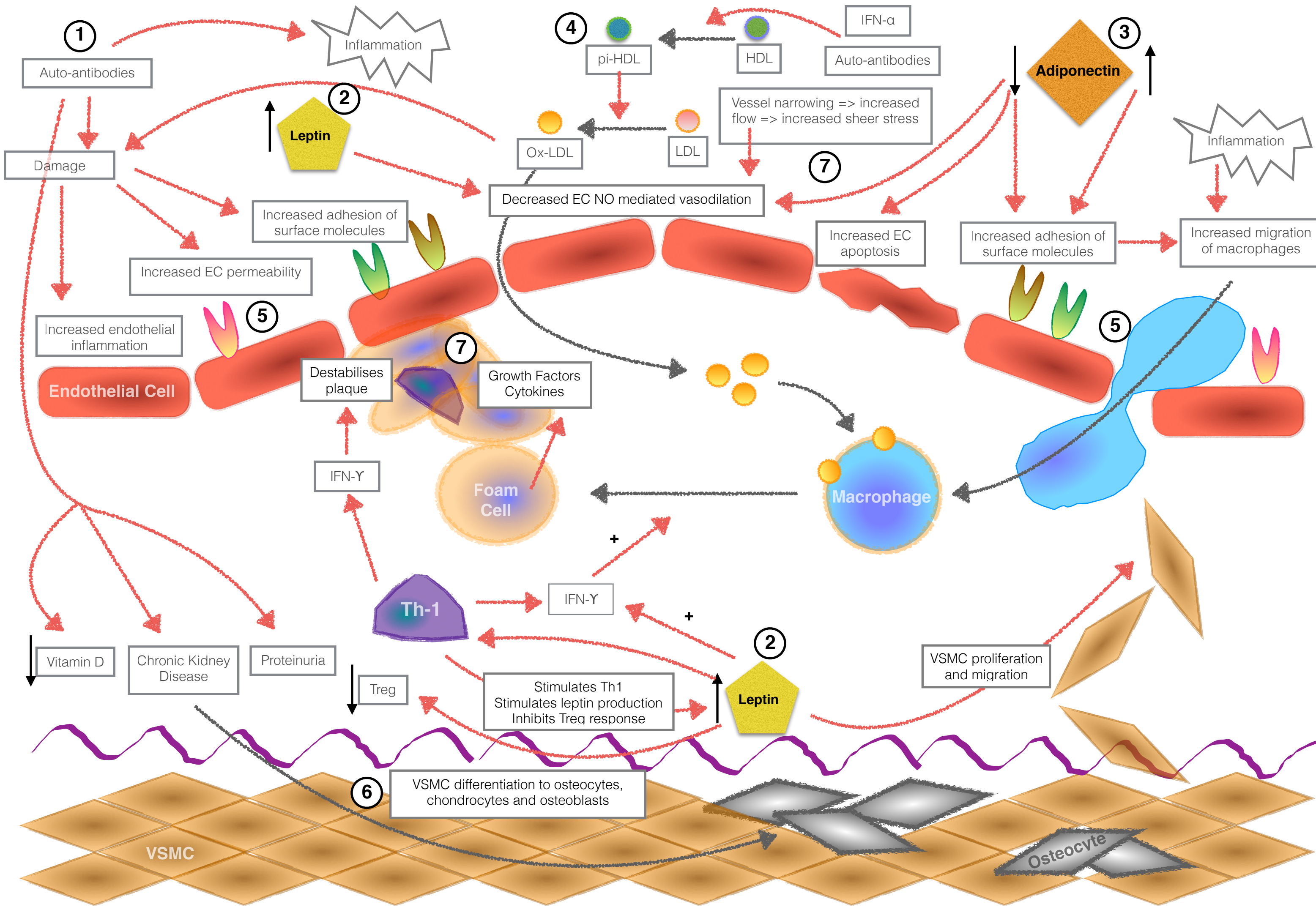
Nonetheless this is a previously undescribed cohort whose findings add to the sparse paediatric literature in this area and the rigorous protocol will enable a follow up study in 5-years, to determine changes over that time. This follow up study should define progression of cIMT and identify whether PWV becomes abnormal over time, along with measurement of FMD to assess endothelial function. It should examine more closely the effect of treatment modalities and renal function on CVD risk while looking at whether quantitative abnormalities in adipokine activity progress over time. An assessment of lipid function would also add significantly to our understanding of the pathogenesis of CVD in JSLE and the use of retinal photography to assess small vessel involvement would also add to our understanding.

In summary, the traditional risk factors for CVD overlap significantly and are individually impacted by SLE thus, while they may appear to cluster in some patients with SLE, it is impossible to fully ascribe the increased risk of CVD to lifestyle risk factors. This cohort of children with JSLE show structural changes in their vessels indicative of early CVD but with adaptive changes resulting in normal functional scans. I have shown a significant difference between ethnicities with a higher cIMT and PWV in the Afro-Caribbean subgroup. Unsurprisingly renal impairment significantly modified the vascular phenotype however even a modest increase in albuminuria appears to be associated with a worsening CV risk profile. Albuminuria also significantly modified the serum adipokine profile, with a doubling of both leptin and adiponectin levels in patients even with a modest UaUc.

**Figure 40. The Unique Pathogenesis of Cardiovascular Disease in Juvenile-onset Systemic Lupus Erythematosus (illustration by C Quinlan)**







# **Publications and Presentations Arising from this Project**



# The vascular phenotype of children with systemic lupus erythematosus

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## Abstract

**Background** The increased risk of cardiovascular disease (CVD) in adults with systemic lupus erythematosus (SLE) has been known since the 1970s, but studies in juvenile-onset SLE (JSLE) have reported conflicting results and more data are needed. The aim of this cross-sectional study was to establish the baseline risk of CVD in a cohort of UK patients with JSLE.

**Methods** Data were collected to establish disease duration, disease activity, medication use and activity levels, as well as demographic data, including family history of CVD. Vascular phenotype was established using well-validated measures of carotid intima media thickness (cIMT) and pulse wave velocity (PWV).

**Results** In total, 45 children (39 female; mean age  $13.5 \pm 2.9$  years) with JSLE were recruited to the study. Of these, 24 had a history of biopsy-proven lupus nephritis and five had an estimated glomerular filtration rate of  $<90$  ml/min/1.73 m<sup>2</sup>. Comparison of these JSLE patients with healthy controls previously scanned at our hospital revealed that the cIMT value was significantly higher in the former (0.45 vs. 0.37 mm, respectively;  $p < 0.0001$ ). This difference was associated with the use of antihypertensives ( $p = 0.04$ ) and higher

or lower doses of prednisolone ( $p < 0.0001$ ). PWV was not significantly different in the patient and control group (5.27 vs. 5.34 m/s, respectively;  $p = 0.77$ ). In the patient group, the mean body mass index percentile was  $65.63 \pm 28.8$ , and the median physical activity score was 1,773 (676–2,854) metabolic equivalents of task (METs). None of the patients admitted to cigarette smoking, and ten had a positive family history of cardiovascular disease (CVD).

**Conclusion** This study shows that our patients with JSLE had increased cIMT without an increase in PWV, suggesting possible early adaptive changes in JSLE. Follow-up data are needed to determine whether these changes result in clinically significant CVD.

**Keywords** Cardiovascular · Lupus · Ethnicity · Physical activity · Lipids · Steroids

## Introduction

Individuals with adult onset systemic lupus erythematosus (SLE) were first identified as having an increased risk of cardiovascular disease (CVD) over 35 years ago by Urowitz et al. [1]. In subsequent years, the increased risk of cardiovascular (CV) events among adults with SLE has been confirmed in a number of prospective studies [2–4] which have demonstrated that individuals with SLE have a higher increased risk of CV events than their unaffected peers.

The increased risk of CVD in chronic inflammatory diseases is well recognised, and several adult studies have shown an abnormal vascular phenotype in SLE. There is evidence to suggest that the change in phenotype may be due not only to disease-specific risk factors (such as severity and duration of disease [5–7], renal impairment [8–10] and medications

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[1, 11–17]) but also to a clustering of traditional risk factors (such as increasing age, obesity, smoking and reduced physical activity [15, 18–20]).

Children with SLE tend to have a more aggressive disease than adults, with a higher incidence of arthritis, lupus nephritis (LN) and neurological involvement [21]. Since disease severity and duration are closely linked to CVD in the adult SLE population, it is reasonable to assume that the paediatric population would similarly be at increased risk of CVD. Despite the wealth of data available on CVD risk in adults with SLE, there is a dearth of information about CVD risk in children with juvenile-onset SLE (JSLE), and most of our knowledge is extrapolated from adult studies. Although childhood lupus is often a more aggressive disease than its adult form, the greater plasticity of children's vessels may protect them from vascular disease, making extrapolations from adult studies difficult; published studies [22–27] to date have reported conflicting results. The cross-sectional study reported here was designed to establish the baseline CV risk of a cohort of children with JSLE prior to subsequent longitudinal follow-up studies. In addition, since children should be free of the classical risk factors for CVD, studying their risk profile may add valuable information to the adult SLE literature.

## Methods

### Recruitment

Children with JSLE, who fulfilled  $\geq 4$  of the American College of Rheumatology classification criteria [28] and who attended the nephrology or rheumatology services in Great Ormond Street Hospital for Children NHS Foundation Trust were invited to take part in the study (referred to as the CV JSLE Cohort Study). Those aged  $< 5$  years and diagnosed with end-stage kidney disease or within 6 weeks of a major surgical procedure were excluded.

The study was approved by the Institute of Child Health and Great Ormond Street Hospital research ethics committee and The Children's University Hospital, Dublin.

### Measurement of carotid artery intima media thickness

In this study carotid artery intima media thickness (cIMT) was measured by B-mode ultrasound of both common carotid arteries using a single ultrasound machine (Vivid7, GE Medical, Horton, Norway) equipped with a 12-MHz linear-array transducer [29]. Following visualisation of the common carotid artery, a 15-heartbeat cine-loop recording was stored for offline analysis using Digital Imaging and Communications in Medicine software (DICOM; National Electrical Manufacturers Association, Rosslyn, VA). The mean IMT of a 0.5- to 1-cm-long segment was measured

1 cm proximal to the carotid bifurcation, using an automated edge detection system, on two separate images of the right and left common carotid artery from true end-diastolic frames, as determined by minimum vessel diameter. Approximately 120 individual measurements were made across a 1-cm length of artery; this measurement was repeated for three different cardiac cycles and the average of the three measurements from right and left was recorded.

### Measurement of pulse wave velocity

Pulse wave velocity (PWV) was determined by using a pressure sensor over the carotid and femoral arteries and calculated as the pulse wave travel distance divided by the time difference between the rise delay of the distal and proximal pulse, as measured by the R wave on the electrocardiogram. In this project, PWV was calculated by SphygmoCor software (AtCor Medical, Sydney, Australia) using the intersecting tangent algorithm. The pulse travel distance was calculated as the distance from the suprasternal notch to the recording point at the femoral artery via the umbilicus minus the distance from the suprasternal notch to the recording point at the carotid artery [30].

### Data collection

A pilot version of the questionnaire was trialed in The Children's University Hospital, Dublin and was used to record demographic data, treatment information and risk factors for CVD. Routine blood samples were processed by the departments of Haematology, Immunology and Clinical Chemistry at Great Ormond Street Hospital for Children NHS Foundation Trust.

Body mass index (BMI) and percentiles were calculated using the Centre for Disease Control and Prevention BMI-for-age growth chart ([www.cdc.gov](http://www.cdc.gov)). A positive family history of CVD was defined as per the Expert Panel on Integrated Guidelines for Cardiovascular health and Risk Reduction [31] as a CV event in a male relative before 55 years of age or in a female relative before 65 years of age. Family history included siblings, parents, uncles, aunts, grand-parents, grand-aunts and grand-uncles. Estimated glomerular filtration rate (eGFR) was calculated by the Schwartz formula with a K value of 34, as calculated locally [32].

### pBILAG–2004 score

Many of the children recruited to this study (CV JSLE Cohort Study) were also co-recruited to the UK JSLE Cohort Study [21]. As part of the protocol the paediatric adaptation of the 2004 British Isles Lupus Assessment Group (pBILAG–2004) disease activity index was measured at 3-month intervals and included in the analysis where available. The pBILAG–2004 index was developed as an intention-to-treat index; it has been



validated in a UK paediatric cohort [33] and shown to be more sensitive than the SLE Disease Activity Index (SLEDAI)–2000 to detect active disease [34]. It is an ordinal scale index with nine systems (Constitutional, Mucocutaneous, Neuropsychiatric, Musculoskeletal, Cardiorespiratory, Gastrointestinal, Ophthalmic, Renal and Haematological) which divides disease activity into five categories where A represents very active disease, B represents moderate disease, C represents mild stable disease, D represents previous disease and E represents no current or previous disease. A continuous total score has been developed and validated [35] and was used in this study to represent systemic disease burden.

## Calculation of physical activity

The International Physical Activity Questionnaire (IPAQ) [36] was used to estimate the physical activity of the study cohort. This short version of this questionnaire was chosen as a well-developed, internationally recognised instrument which enables scoring of the cohort by metabolic equivalents of task (MET). This questionnaire proposes three levels of physical activity: “low” where there is no activity or <600 MET min/week are recorded; “moderate” where 600–1500 MET min/week are recorded; “high” where >1,500 MET min/week are recorded. This scoring protocol was recently used by Katz et al. [37] to show that 28 % of 138 women with SLE with a mean age of 18 years scored <600 MET min/week.

## Statistics

For normally distributed data results are reported as the mean  $\pm$  standard deviation (SD), and comparisons between means were made using unpaired *t* test results (2-tailed). For non-normally distributed data, results are reported as the median  $\pm$  interquartile range (IQR), and comparisons between medians were made using the Mann–Whitney test. Correlations were made using Pearson’s correlation co-efficient or Spearman’s test depending on data distribution.

Within this project a *p* value <0.05 was determined to have reached significance. Statistical analyses were performed in GraphPad Prism version 6.0b for Mac OS X, and multiple regression analysis was performed using GraphPad InStat version 3.10 32bit for Windows (GraphPad Software, La Jolla, CA; [www.graphpad.com](http://www.graphpad.com)).

## Results

### Demographics

Forty-five children were recruited to our study, among whom 39 were girls. The median age of the children was 9.75 (IQR 7.23–12.6) years at diagnosis and 14 (IQR 12.2–15.5)

years at recruitment. Forty-four consented to have all vascular scans performed, and one patient was unable to remain sufficiently still for the PWV scanning. Thirty-five children of these children were also recruited to the UK JSLE Cohort Study; pBILAG-2004 scores were calculated and vascular assessments were performed for 28 of these children [Electronic Supplementary Material (ESM)]. The control group comprised subjects who had participated in previous studies [38] from our centre and who were matched for historical age. Demographic details of patients and controls are outlined in Table 1.

Nineteen children identified themselves as Afro-Caribbean, of whom 13 identified themselves as of African descent, two as Black British and four as Black Caribbean. Thirteen children identified themselves as Caucasian and ten as Asian (5 Indian, 1 Pakistani 1 Chinese, 3 “other Asian”). One child was

**Table 1** Demographic, clinical and anthropometric characteristics of children and young adults with juvenile-onset systemic lupus erythematosus and controls

Demographic, clinical and anthropometric characteristics	Children with JSLE ( <i>n</i> =45)	Controls ( <i>n</i> =40)	
Age (years)	14 (12.2–15.5)	13.2 $\pm$ 4.8	t1.1
Female ( <i>n</i> )	39	19	t1.2
Weight (kg)	50.5(36.5–62)		t1.3
Height (cm)	150.1 $\pm$ 16		t1.4
BMI	21.63 (18.19–24.57)		t1.5
BMI percentile	65.63 $\pm$ 28.8		t1.6
Biopsy-proven lupus nephritis ( <i>n</i> )	24		t1.7
Disease duration (months)	39 (20.5–64.5)		t1.8
Age at diagnosis (months)	115 $\pm$ 42		t1.9
Cigarette smoking ( <i>n</i> )	0		t1.10
Oral contraceptive pill ( <i>n</i> )	1		t1.11
Positive family history of CVD ( <i>n</i> )	11		t1.12
Current prednisolone (mg/kg)	0.07 (0–0.23)		t1.13
No prednisolone ( <i>n</i> )	16		t1.14
Normotensive without anti-hypertensive medication ( <i>n</i> )	26		t1.15
Enalapril ( <i>n</i> )	12		t1.16
Amlodipine ( <i>n</i> )	8		t1.17
Atenolol ( <i>n</i> )	2		t1.18
Furosemide ( <i>n</i> )	2		t1.19
Irbesartan ( <i>n</i> )	1		t1.20
METs/week	1,773 (676.5–2,854)		t1.21
Sitting (min/day)	505 $\pm$ 144.2		t1.22
Mean cIMT (mm)	0.445 (0.42–0.49)	0.37 $\pm$ 0.06	t1.23
Mean PWV (m/s)	5.272 $\pm$ 0.88	5.34 $\pm$ 0.97	t1.24

Data are presented as the mean  $\pm$  standard deviation (SD) or as the median with the interquartile range (IQR) in parenthesis, unless indicated otherwise

JSLE, Juvenile-onset systemic lupus erythematosus; BMI body mass index; CVD, cardiovascular disease; METS, metabolic equivalent of task; cIMT, carotid intima media thickness; PWV, pulse wave velocity

220 Afghani, one was Turkish Cypriot and one declined to answer  
221 this question. The ethnic composition of our patient group dif-  
222 fers from that of the overall UK JSLE Cohort, as outlined in  
223 Table 2, where the majority of children were Caucasian and  
224 almost one-third were Black Afro-Caribbean. By contrast, of  
225 the 45 children enrolled in our study (CV JSLE Cohort Study in  
226 Table 2), 19 were Black Afro-Caribbean and 13 were  
227 Caucasian, which reflects the ethnic diversity of London rela-  
228 tive to the rest of the UK.

229 Median disease duration was 39 (IQR 20.5–64.5) months.  
230 Disease activity was assessed using the pBILAG–2004 dis-  
231 ease activity score within 3 months of recruitment. The medi-  
232 an pBILAG-2004 score was 3.5 (IQR 1.25–5.75), which is in  
233 line with that of the treated national cohort group (UK JSLE  
234 Cohort Study) which had a median pBILAG–2004 score at  
235 12 months after diagnosis of 2 (IQR 1–4).

## 236 Traditional CV risk factors

237 No children admitted to cigarette smoking, and one child had  
238 started taking the oral contraceptive pill 1 month previous to

**Q2 t2.1 Table 2** Comparison of patient demographic data for the UK Juvenile-onset Systemic Lupus Erythematosus (JSLE) Cohort Study and the CV JSLE study

t2.2	Demographic characteristics	UK JSLE Cohort Study	CV JSLE Cohort Study
t2.3	Total number of children	196	45
t2.4	Gender		
t2.5	Female	168 (85)	39 (87)
t2.6	Female:male	5.1:1	6.5:1
t2.7	Age		
t2.8	At diagnosis (years)	12.6	9.6
t2.9	At analysis (years)	17.4	13.5
t2.10	Ethnicity		
t2.11	Caucasian	103 (52)	13 (29)
t2.12	Black Afro-Caribbean	29 (15)	19 (42)
t2.13	Other Asian	12 (6)	3 (7)
t2.14	Indian	18 (9)	5 (11)
t2.15	Pakistani	12(6)	1(2)
t2.16	Anti-hypertensive use		
t2.17	Angiotensin-converting enzyme inhibitor (ACEi)	48 (24)	12 (27)
t2.18	Angiotensin receptor blocker (ARB)	24 (12)	1 (2)
t2.19	Renal function		
t2.20	pBILAG-2004 (A–D)	141/176 (80)	25/28 (89)
t2.21	Proteinuria	7(3.5)	12 (27)
t2.22	Estimated glomerular filtration rate (eGFR) <50 %	1 (0.5)	0

Data are presented as the number with the percentage in parenthesis  
pBILAG-2004, Paediatric British Isles Lupus Assessment Group disease  
activity index

recruitment. Ten children had a positive family history of  
CVD as defined by Expert Panel on Integrated Guidelines  
for Cardiovascular health and Risk Reduction.

The mean serum cholesterol level was 4.4 (IQR 3.95–5.2) mmol/l, serum triglycerides were 1.04 (0.76–1.93) mmol/l, serum high-density lipoprotein (HDL) was 1.28±0.46 mmol/l, low-density lipoprotein (LDL) was 2.35 (2.14–2.99) mmol/l and very-low-density lipoprotein was 0.64±0.4556 mmol/l. Nine children had a serum cholesterol >5 mmol/l, eight had a serum HDL of <1 mmol/l and four had a serum LDL of >3.5 mmol/l.

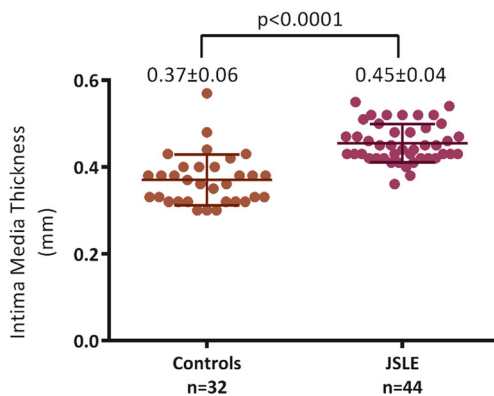
For those children with a serum cholesterol of >5 mmol/l, mean cIMT was 0.47±0.05 mm and mean PWV was 5.49±0.7 m/s; for those with a serum cholesterol of <5 mmol/l, mean cIMT was lower at 0.45±0.04 mm and PWV was also lower at 5.24±0.8 m/s. Neither result reached statistical significance. Abnormalities in serum lipids were correlated with duration of disease, younger age of diagnosis, mean cIMT, PWV, eGFR, increased systolic blood pressure (SBP) and increased BMI. Those children with a serum cholesterol of >5 mmol/l were on a lower mean dose of prednisolone (0.26±0.27 mg/kg) than those with a serum cholesterol of <5 mmol/l (4.28±5.9 mg/kg) ( $p=0.054$ ), possibly indicating that higher cholesterol was associated with disease activity rather than secondary to medications.

Mean BMI percentile was 65.63±28.8. The median physical activity score was 1773 (IQR 676–2854) METs, which correlated with duration of disease ( $r^2=0.33$ ,  $p=0.04$ ). The median physical activity score was 1,773 (IQR 676–2,854) METs, which correlated with duration of disease ( $r^2=0.325$ ,  $p=0.038$ ). These results correspond to a high level of activity, but there was significant heterogeneity within the group. Interestingly, children with a positive family history of CVD had a higher median MET score than those with no family history of CVD [1,738 (IQR 677–2,741) vs. 568 (IQR 0–1,689) METs, respectively], but this difference did not reach statistical significance. Not surprisingly, there was a strong correlation between BMI percentile and physical activity. The median BMI percentile for children with a low MET score, i.e. <600 MET, was 88.2 (IQR 49.9–97.5) and for those with a high MET score, i.e. >1500 MET, it was 65.8 (32.5–85.5); this difference did not reach statistical significance.

## Vascular phenotype

### *Carotid intima media thickness*

Forty-four children with JSLE had cIMT measurements; the control group consisted of 40 patients. Mean cIMT for the control group and JSLE group was 0.37±0.06 and 0.45±0.04 mm, respectively (Fig. 1). Children with JSLE had a significant increase in mean cIMT of 0.08 mm when compared with the controls ( $p<0.0001$ .) There was no statistically



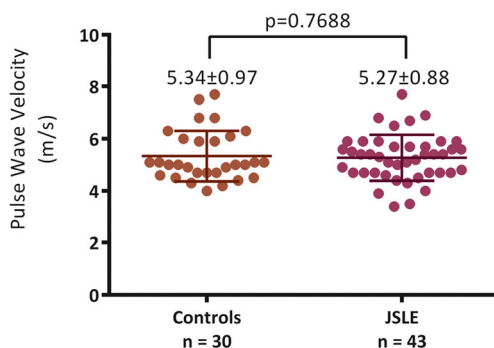
**Fig. 1** Comparison of carotid intima media thickness (cIMT) between controls and patients with juvenile-onset systemic lupus erythematosus (JSLE)

significant association between cIMT and upper or lower quartiles of age, METs, pBILAG–2004 score, pBILAG–2004 renal score or disease duration. An increase in cIMT was also not associated with male gender or a family history of CVD. However, cIMT did correlate weakly with the number of sitting min/week ( $r^2=0.234$ ,  $p=0.176$ ) and BMI percentile ( $r^2=0.276$ ,  $p=0.077$ ).

#### Pulse wave velocity

Forty-three children with JSLE had PWV measurements; the control group consisted of 40 children. Mean PWV for the control and patient groups was  $5.34\pm0.97$  and  $5.27\pm0.88$  m/s, respectively (Fig. 2). Children and young adults with JSLE had a 1.2 % lower PWV than the controls, but this difference was not statistically significant ( $p=0.77$ ). Increased PWV was associated with disease duration ( $r^2=0.37$ ,  $p=0.02$ ), age ( $r^2=0.35$ ,  $p=0.02$ ), SBP ( $r^2=0.35$ ,  $p=0.02$ ), LDL ( $r^2=0.37$ ,  $p=0.048$ ) and eGFR ( $r^2=0.331$ ,  $p=0.06$ ).

There was no statistically significant association between PWV and the upper or lower quartiles of age, pBILAG–2004 score, pBILAG–2004 renal score, prednisolone dose, METs or disease duration. An increase in PWV was also not associated with male gender or a family history of CVD (Table 3).



**Fig. 2** Comparison of pulse wave velocity (PSV) between controls and patients with JSLE

#### Renal function and hypertension

All 45 JSLE patients were normotensive at the time of the study. Twenty-six were not taking any anti-hypertensive medication, and 15 were on a single medication [enalapril (9 patients), amlodipine (4) furosemide (1) and irbesartan (1)], two were on two medications, one was on three medications and one patient required four medications to maintain normal blood pressure.

There was a significant association between the use of anti-hypertensives and an increase in cIMT. The cIMT in the normotensive group not treated with antihypertensives and in the normotensive group treated with antihypertensives was  $0.45\pm0.04$  and  $0.47\pm0.05$  mm, respectively ( $p=0.04$ ). The relationship between antihypertensive use and cIMT remained significant even after controlling for total pBILAG–2004 score, prednisolone dose, age, family history of CVD and gender ( $p=0.04$ ). In the absence of antihypertensive medication, the mean cIMT for patients with JSLE was significantly higher than that of controls at  $0.45\pm0.04$  mm ( $p<0.0001$ ). (see Fig. 3).

There was a significant association between the use of anti-hypertensives and an increase in PWV. The PWV in the normotensive and anti-hypertensive groups was  $5.13\pm0.6$  and  $5.69\pm1.13$  m/s, respectively ( $p=0.043$ ). Similar to the cIMT findings, the PWV was increased in these children compared to the controls, even in the absence of anti-hypertensives, from  $5.34\pm0.97$  to  $5.13\pm0.06$  m/s; however this difference was not statistically significant ( $p=0.132$ ).

Those children defined as hypertensive may only have had a low level of hypertension (HTN) and proteinuria. The median urine albumin-to-creatinine ratio (UaUc) for the JSLE cohort was 2.9 (IQR 1.2–55.3) mg/mmol. In an effort to assess the effect of renal dysfunction on the vascular phenotype we examined only those children with a grossly elevated UaUc ( $>20$  mg/mmol) and compared their vascular phenotype with both the group as a whole and with a those with a UaUc closer to the normal range (Table 3). The median UaUc of the two subgroups were similar to that of the overall cohort with regards to age, gender, disease duration and BMI. Not unsurprisingly, the children with elevated UaUc were significantly more likely to be concurrently treated with antihypertensive medication, most commonly an angiotensin-converting enzyme inhibitor (ACEi), which was used as much for the management of proteinuria as for the management of HTN.

With regards to their vascular phenotype, children with an elevated UaUc had an elevated cIMT [ $0.455$  (IQR  $0.43$ – $0.49$ ) vs.  $0.435$  ( $0.42$ – $0.49$ ) mm] and a prolonged PWV ( $5.564\pm0.72$  vs.  $5.172\pm0.92$  m/s). However, the numbers were too small to reach statistical significance.

Median eGFR was  $103.3$  (IQR  $95.65$ – $113.3$ ) ml/min/ $1.73$  m<sup>2</sup>. Five children had an eGFR of  $<90$  ml/min/ $1.73$  m<sup>2</sup> ( $26.3$ ,  $34.5$ ,  $66.8$ ,  $84.6$  and  $87.2$  ml/min/ $1.73$  m<sup>2</sup>, respectively).



**Table 3** Demographic, clinical and anthropometric characteristics of children and young adults with JSLE and a normal or grossly elevated urine albumin-to-creatinine ratio

Demographic, clinical and anthropometric characteristics	Children with JSLE (n=45)	UaUc <20 mg/mmol (n=33)	UaUc >20 mg/mmol (n=12)
UaUc (mg/mmol)	2.9 (1.2–55.3)	1. (0.95–3.5)	164.6 (55.3–304.9)
Age (months)	162±35	160±38	165±20
Gender (♀:♂)	39:6	29:4	10:2
BMI	21.6 (18.2–24.6)	21.3 (18.2–24.3)	22.29 (18.7–25.2)
BMI centile	65.63±28.8	63.7±29	70.5±28.8
Disease duration (months)	39 (20.5–64.5)	22 (21.8–71)	36.5 (7.5–49.5)
Positive family history of CVD	11 (24.4 %)	9 (27.3 %)	2 (16.6 %)
Treatment for hypertension	16 (35.5 %)	9 (27.3 %)	8 (66.7 %)
METS/week	1,773 (676.5–2,854)	2,190 (1,127–3,306)	886 (615–1,648)
Median cIMT (mm)	0.445 (0.42–0.49)	0.435 (0.42–0.495)	0.455 (0.43–0.49)
Mean PWV (m/s)	5.272±0.88	5.172±0.92	5.564±0.72

Data are presented as the mean±SD or median with the IQR in parenthesis, unless indicated otherwise

UaUc, Urine albumin-to-creatinine ratio

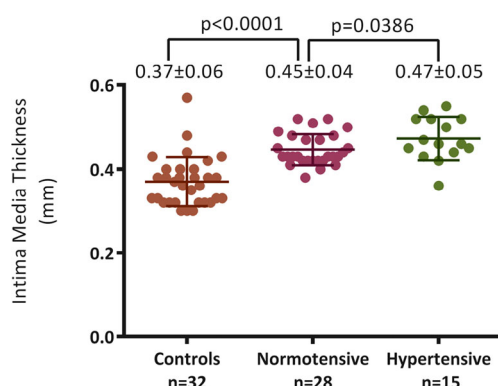
Median cIMT for the children with an eGFR of <90 ml/min/1.73 m<sup>2</sup> ml/min/1.73 m<sup>2</sup> and for those with an eGFR of <70 ml/min/1.73 m<sup>2</sup> ml/min/1.73 m<sup>2</sup> was 0.45 (IQR 0.4–0.5) and 0.45 (IQR 0.36–0.55) mm, respectively. There was no difference when compared to children with JSLE and an eGFR >90 ml/min/1.73 m<sup>2</sup>. The difference between medians was not significant ( $p=0.556$ ). However, median PWV for children with an eGFR of <90 ml/min/1.73 m<sup>2</sup> and those with an eGFR of <70 ml/min/1.73 m<sup>2</sup> was 5.7 (IQR 5.5–6.6) and 6.8 (IQR 5.7–6.9) m/s, respectively. This is an increase of 1.1 m/s when compared to children with an eGFR of >90 ml/min/1.73 m<sup>2</sup> and JSLE, and this difference did reach statistical significance despite the small numbers ( $p=0.0277$ ).

Renal involvement was also assessed using the pBILAG–2004 score for those who had performed this test, and the data are summarised in Table 4. One child was diagnosed with severe renal disease activity (category A), five were diagnosed with moderate renal disease activity (category B), five were diagnosed with stable renal disease activity (category C), 16 were diagnosed with currently quiescent but previously active

renal disease (category D) and only three of the 30 children tested were defined as no renal involvement ever (category E). Those with current LN (categories A/BC) had slightly increased cIMT ( $0.46\pm0.06$  mm) compared with those with quiescent LN (categories D/E;  $0.45\pm0.04$  mm) ( $p=0.73$ ). Similarly, there was little change in the PWV ( $5.46\pm0.95$  vs.  $5.43\pm0.74$  m/s;  $p=0.78$ ).

#### Prednisolone and the vascular phenotype

There was significant increase in cIMT between controls and patients receiving no prednisolone, and those receiving the lower and upper quartiles of prednisolone (0.087 and 0.315 mg/kg, respectively). The cIMT of those receiving no prednisolone measured  $0.43\pm0.04$  mm, which increased to  $0.5\pm0.04$  mm for those on low doses and to  $0.45\pm0.05$  mm for those on higher doses. There was a significant difference in cIMT between controls and those on all doses of prednisolone ( $p<0.05$ ) and between those on no prednisolone and the lower quartile ( $p=0.0018$ ) (Fig. 4).



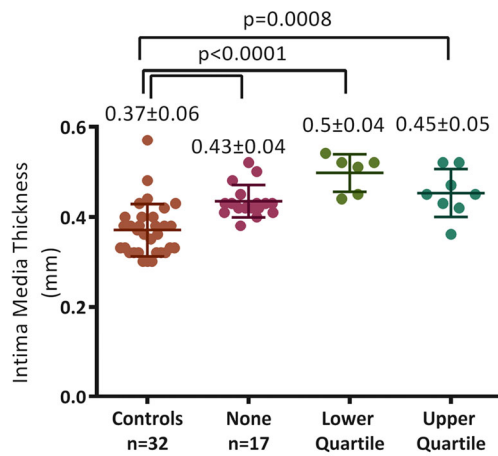
**Fig. 3** Comparison of cIMT between controls and patients with JSLE according to use of anti-hypertensive medication

**Table 4** Pulse wave velocity and carotid intima media thickness by the pBILAG–2004 score

pBILAG renal score <sup>a</sup>	n	Mean cIMT	Mean PWV
A/B	5	0.45±0.07 mm	5.7±1.09 m/s
C	5	0.48±0.04 mm	5.18±0.88 m/s
D	14	0.46±0.05 mm	5.24±0.79 m/s
E	3	0.44±0.02 mm	5.43±0.95 m/s

All data are presented as the mean±SD

<sup>a</sup> PBILAG–2004 categories: A, very active disease; B, moderate disease; C, mild stable disease; D, previous disease; E, no current or previous disease

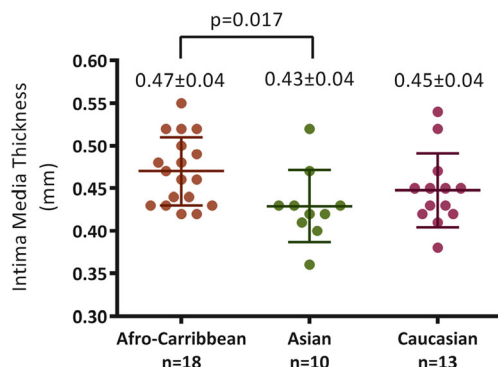


**Fig. 4** Comparison of cIMT between controls and patients with JSLE according to daily dose of prednisolone [quartiles: none lower, higher (mg/kg/day)]

#### Ethnicity and the vascular phenotype

When analysed by ethnicity there was a significant difference between the Asian population and both the Caucasian and Afro-Caribbean population, despite the small overall numbers. One Caucasian child was unable to remain still for the PWV reading and one Afro-Caribbean child declined to consent to vascular scanning. Mean cIMT was highest in the Afro-Caribbean cohort ( $0.47 \pm 0.04$  mm) and lowest in the Asian cohort ( $0.429 \pm 0.04$  mm); the Caucasian cohort had a median cIMT of  $0.448 \pm 0.04$  mm. This difference was statistically significant for the Afro-Caribbean cohort compared to the Asian cohort ( $p=0.017$ ), but it did not meet statistical significance when comparing the other cohorts (Fig. 5).

There was a similar difference noted among the PWV results. Mean PWV was highest for the Afro-Caribbean cohort ( $5.339 \pm 0.74$  m/s) but lowest for the Caucasian population ( $5.225 \pm 1.148$ ). The high standard deviation suggests greater spread amongst this group. Mean PWV among the Asian cohort was  $5.31 \pm 0.69$  m/s. None of these comparisons reached statistical significance.



**Fig. 5** Comparison of cIMT between patients with JSLE erythematosus by ethnic background

#### Discussion

There is strong epidemiological evidence to show that adult onset SLE is associated with an increased risk of clinical and preclinical CVD. However, the increased prevalence of traditional risk factors in this cohort, such as smoking and obesity, makes it difficult to study the effect of SLE-specific factors and interventions, since many SLE patients will already have a significant lifetime CV risk due to non-modifiable risk factors, such as a lifetime of smoking. The aim of our study was to establish the risk of CVD in a population free from these traditional risk factors and, thereby, perform a closer analysis of disease-specific risk factors.

Patients with JSLE generally have more severe disease, with a greater prevalence of LN [21], and will have a longer lifetime burden of disease. To date, the results of published studies examining CVD in JSLE have been conflicting, and many of the published studies have lacked control groups and thus relied on the correlation of risk factors with changes in vascular measures.

cIMT is a measurement of the intima and media layers lining the carotid artery. It is a measure of very early structural changes in the artery and an important surrogate marker for atherosclerosis. Carotid-femoral PWV is a measurement of the time delay between the foot of the pulse pressure arriving at the carotid and femoral arteries, and it is a widely used method to determine arterial stiffness [39, 40]. This cohort of children and adolescents with SLE and normal renal function have an increased cIMT, i.e. a structural change in the vessel wall, but without an increase in PWV, which is a functional measurement of vessel health. Indeed, the median PWV was lower in our JSLE cohort than in the controls, suggesting alternate pathways for the development of CVD in JSLE or a degree of compensation in the early stages of SLE, which may be overwhelmed in time.

In a study designed to assess the effect of atorvastatin on the progression of subclinical atherosclerosis, as measured by cIMT, Schanberg et al. [22, 24] found a mean cIMT of 0.468 mm, similar to our data. Increased cIMT was associated with both a high and low dose of prednisolone, BMI, increased age and creatinine clearance. This study included children with mildly active disease (mean SLEDAI 4.6), with 36 % having LN and 34 % having HTN. In contrast, our patients showed a greater prevalence of active LN. Secondary analysis of the data reported by Schanberg et al. [22, 24] showed that atorvastatin may slow the progression of cIMT thickening only in a subgroup of pubertal patients with higher high-sensitivity C-reactive protein [23]. In contrast to our findings, Boros et al. [27] found an increase in PWV, but no increase in cIMT or flow-mediated dilatation, among a cohort of children with SLE. Although these authors showed an increased burden of traditional and non-traditional CV risk factors among the cohort, such as HTN, these did not correlate

with changes in vascular measures. When subdivided by disease activity, El Gamal et al. [26] showed increased PWV in hypertensive patients with active SLE when compared to controls, but no difference between controls and patients with inactive disease as defined by SLEDAI.

Interestingly, Sozeri et al. [25] showed significantly increased cIMT and PWV in patients with lupus when compared with controls. In this study, cIMT correlated with disease activity but not with HTN or LN. However, in the ten patients (20 %) with HTN (8 of whom had left ventricular hypertrophy), the authors found an increased PWV; they also showed a marginal increase in PWV in those with biopsy-proven LN when compared to those with normal renal biopsies.

The increase in cIMT in our cohort was most marked in the patients treated with antihypertensives. Since the commonest antihypertensive in use was an ACEi, which is also used to control proteinuria, the difference between groups may be explained by renal involvement or severity of disease. However, there we found no correlation between cIMT or PWV and either the overall or the renal pBILAG-2004 score, or indeed the prednisolone dose, which could be presumed to be a reasonable surrogate marker for disease severity.

In this study, we found no difference in BMI percentiles between patients and controls, a low prevalence of sedentary behavior and no effect of smoking. Indeed, in contrast to their adult counterparts [37], this cohort of children with JSLE showed high levels of activity, with a median of 1,773 METs per week, corresponding to a high level of physical activity. Encouraging an active lifestyle during clinical encounters is one way in which paediatricians could potentially reduce their patients' future burden of CVD.

Disease duration has been shown to increase the risk of a CV event in adults with SLE and has been associated with subclinical CVD as measured by coronary calcium scores [6, 7, 41–46]. Since the length of disease is correlated with CV risk, there is justifiable concern that children, whose disease has an earlier onset, will be at an increased risk as they age.

The difference in vascular phenotype between ethnicities is similar to that found in previous studies which showed that the presence of carotid plaque was higher in African American women with SLE compared with Caucasian women in contrast to studies of non-SLE subjects [47]. Likewise, Ghosh et al. observed increased fibromuscular dysplasia (FMD) and cIMT in 60 Indian patients with SLE who showed impaired FMD and abnormal IMT [48]. Our group has a greater proportion of African American patients than other paediatric studies [22–27], and this may have affected the results.

The authors acknowledge a number of limitations of this study: the numbers are small, the patient cohort is heterogeneous and the controls were historical. Nonetheless, this is a previously undescribed cohort taken from the largest referral centre for JSLE in the UK and we used a rigorous study

protocol which will enable long-term follow up to determine if the increase in surrogate markers for CVD will result in clinical outcomes.

In summary, this study describes early structural changes in the blood vessels of children and adolescents with JSLE, which may be related to ethnicity and renal involvement and which are associated with CVD in adults. The patients in our study cohort had minimal modifiable risk factors and were physically active, limiting the potential for lifestyle advice. Patients should be made aware of this potentially increased risk as they grow up, and efforts should be made to encourage healthy lifestyle choices in order to decrease their CV risk from traditional, modifiable risk factors. Follow-up data from this cohort are needed to determine if the early compensatory changes resulting in normal PWV readings are overwhelmed with time and if the increase in pre-clinical markers of CVD are followed by hard CV endpoints.

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# Are children with lupus at increased risk of cardiovascular disease

Quinlan C<sup>1,2,3,4</sup>, Kari J<sup>2,4</sup>, Marks S<sup>2,4</sup>, Tullus K<sup>2,4</sup>.



1. The Royal Children's Hospital, Melbourne. 2. Great Ormond Street Hospital for Children NHS Trust, London. 3. The Children's University Hospital Dublin. 4. The Institute of Child Health, London

## Background

SLE is an autoimmune disease, affecting multiple organ systems and leading to a relapsing and remitting chronic inflammatory state. Over the past 2 decades it has become clear that CVD is in itself an inflammatory process and that individuals with chronic inflammatory disease, such as SLE or rheumatoid arthritis, are at increased risk. JSLE is associated with a more aggressive phenotype and a higher prevalence of renal involvement (up to 80%) than adult-onset SLE. As children with diseases such as chronic kidney disease (CKD) have been shown to have a risk of CVD similar to their adult counterparts so clinicians have worried that paediatric patients with SLE are at increased risk of CVD.

## Hypotheses

1. JSLE is associated with an increased risk of CVD and that this is associated with an abnormal vascular phenotype.
2. Children with JSLE have a low prevalence of the classical modifiable risk factors for CVD, obesity, inactivity and smoking, and thus represent a “clean” population in which to study the effect of SLE and the treatment of SLE on the vascular phenotype.
3. The increased risk of CVD in JSLE is related to increased disease activity leading to abnormalities in serum lipids and adipokine activity followed by the development of an abnormal vascular phenotype.
4. The increased risk of CVD in JSLE is further exacerbated by the presence of hypertension, renal impairment, the use of corticosteroids and the duration of disease.

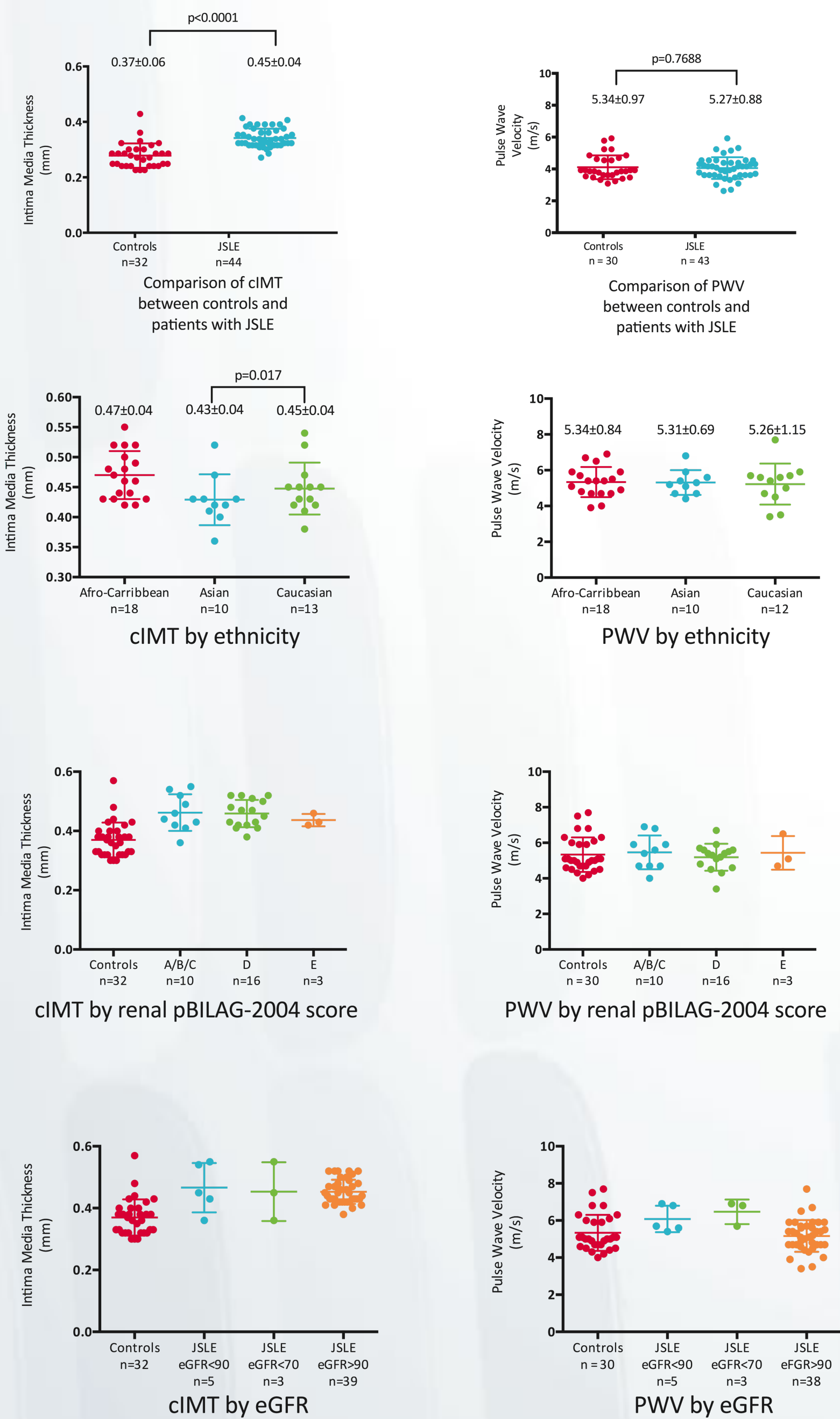
## Methods

Data was collected to establish disease duration, disease activity, medication use and activity levels as well as demographic data including family history of CVD. Vascular phenotype was established using well-validated measures of carotid intima media thickness (cIMT) and pulse wave velocity (PWV).

## Demographic Details

	Children with JSLE (n=45)
Age (months)	162±35
Gender (♀:♂)	39:6
BMI centile	65.63±28.8
Disease duration (months)	39(20.5-64.5)
Age at diagnosis (months)	115 ±42
Cigarette smoking	0
Oral contraceptive pill	1
Positive family history of CVD	11
METS/week	1773(676.5-2854)
Sitting minutes/day	505±144.2
Mean cIMT (mm)	0.445(0.42-0.49)
Mean PWV (m/s)	5.272 ±0.88

## Results



## Conclusion

While this study does not offer conclusive evidence of an increased risk of CVD in UK-based patients with JSLE, it is suggestive of early structural changes, which may be related to ethnicity and renal involvement, and which could become clinically significant with time. Interestingly this occurred without an increase in PWV, a functional measurement of vessel health. Indeed, the median PWV was lower in the JSLE cohort than in controls, suggesting alternate pathways for the development of CVD in JSLE or a degree of compensation in the early stages of SLE, which may be overwhelmed in time. Patients should be made aware of the potential increased risk as they grow up and efforts should be made to encourage healthy lifestyle choices in order to decrease their CV risk from traditional, modifiable risk factors. Follow-up data from this cohort is needed to determine if the early compensatory changes resulting in normal PWV readings are overwhelmed with time and if the increase in pre-clinical markers of CVD are followed by hard CV endpoints.



# Serum Adipokines and Vascular Phenotype in Juvenile-onset Systemic Lupus Erythematosus

Quinlan C<sup>1,2,3,4</sup>, Petrasca A<sup>5</sup>, Kari J<sup>2,4</sup>, Marks S<sup>2,4</sup>, Tullus K<sup>2,4</sup>.



1. The Royal Children's Hospital, Melbourne. 2. Great Ormond Street Hospital for Children NHS Trust, London. 3. The Children's University Hospital Dublin. 4. The Institute of Child Health, London. 5. Trinity College, Dublin.

## Background

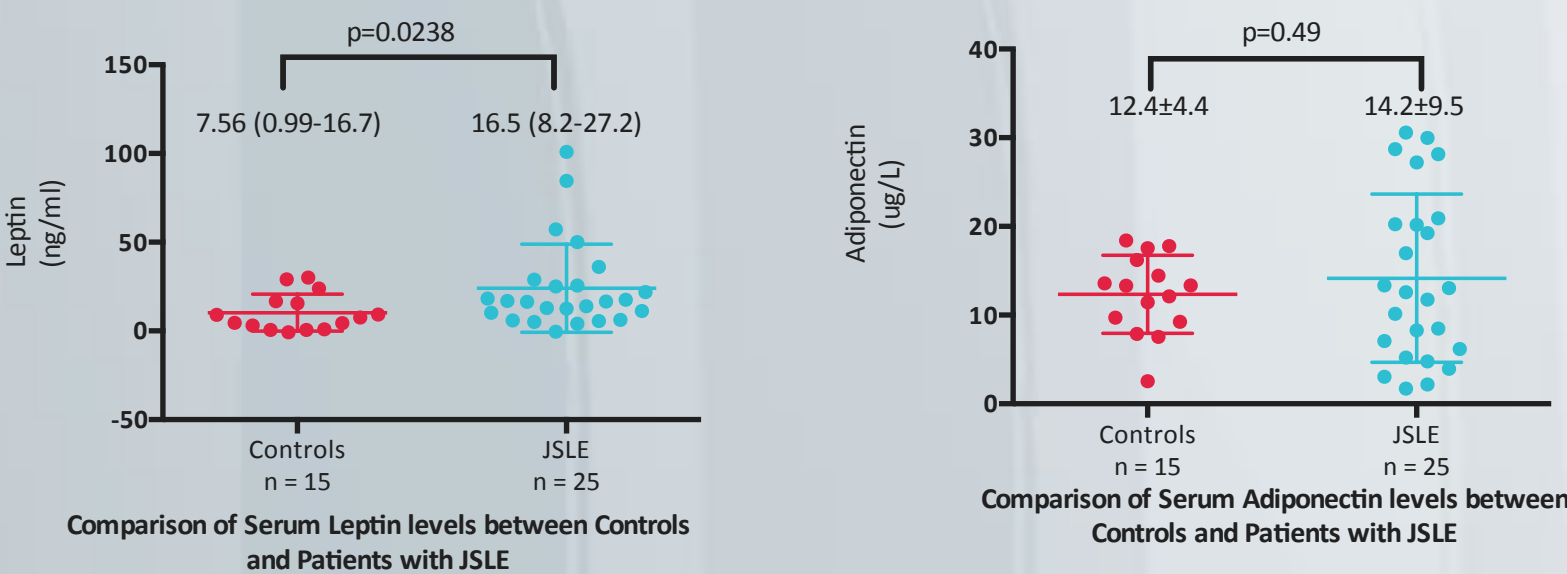
Obesity is a well-established traditional CV risk factor and affects up to 50% of adults with SLE. Over the last decade there has been a shift in thinking about adipose tissue in the last decade – rather than being considered merely a passive storage place for energy it is now recognised as an active endocrine organ. Obesity is a chronic inflammatory state and likely has many similarities with auto-immune disease.

White adipose tissue interacts with the vasculature through the secretion of adipokines predisposing the individual to inflammation leading to atherosclerosis. Leptin levels are increased in inflammation and autoimmune disease. It has long been known to modulate bone mass, however more recently it has also been shown to affect VSMC proliferation, leading to plaque destabilization and increased thrombosis. Adiponectin acts to protect the vasculature from atherosclerosis decreasing endothelial cell apoptosis and reducing the adhesion of surface molecules thus reducing the endothelial injury, which can initiate an atherosclerotic plaque. It defends the VSMC against injury and decreases VSMC migration, reducing the migration of macrophages and their transformation into foam cells.

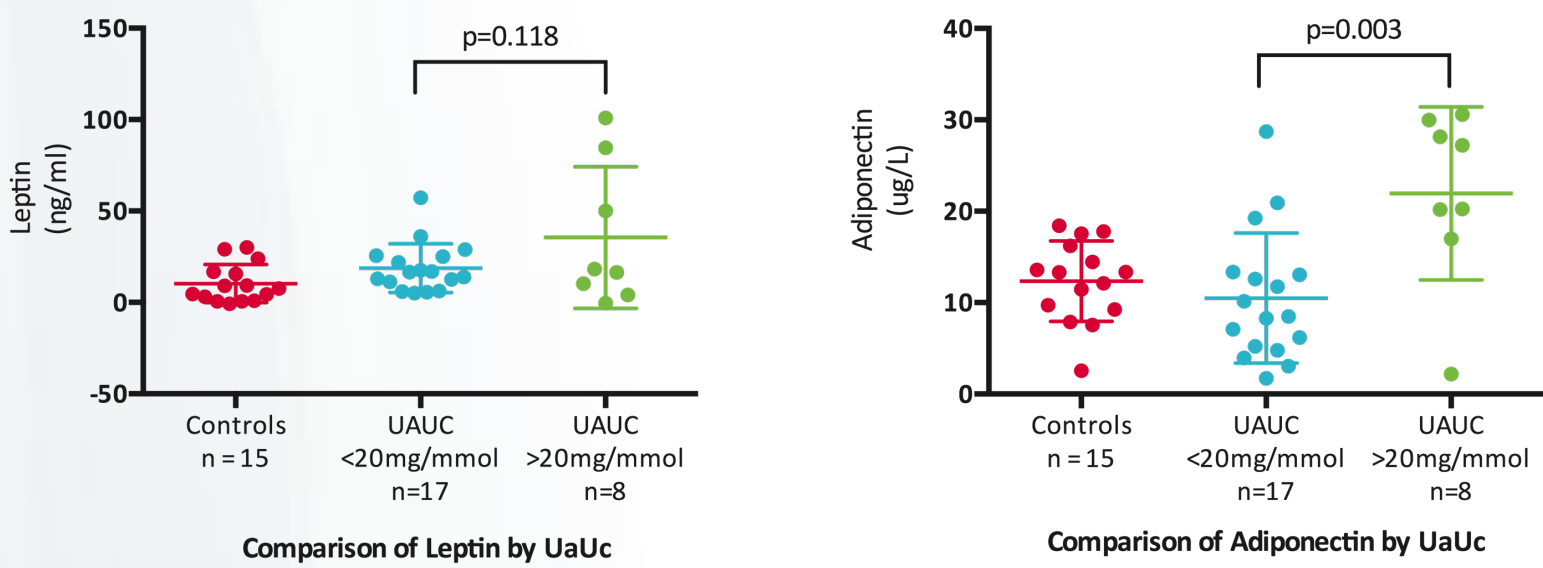
## Methods

Children with JSLE were recruited from the nephrology and rheumatology departments in Great Ormond Street Hospital for Children NHS Trust, the largest paediatric centre in the UK caring for children with JSLE. Controls for the adipokine assays were recruited from healthy children in The Children's University Hospital, Dublin. The following was recorded: demographic details, physical activity score, routine bloods, physical exam and blood pressure. The following was assessed: carotid intima media thickness, pulse wave velocity, serum leptin and serum adiponectin.

## Results

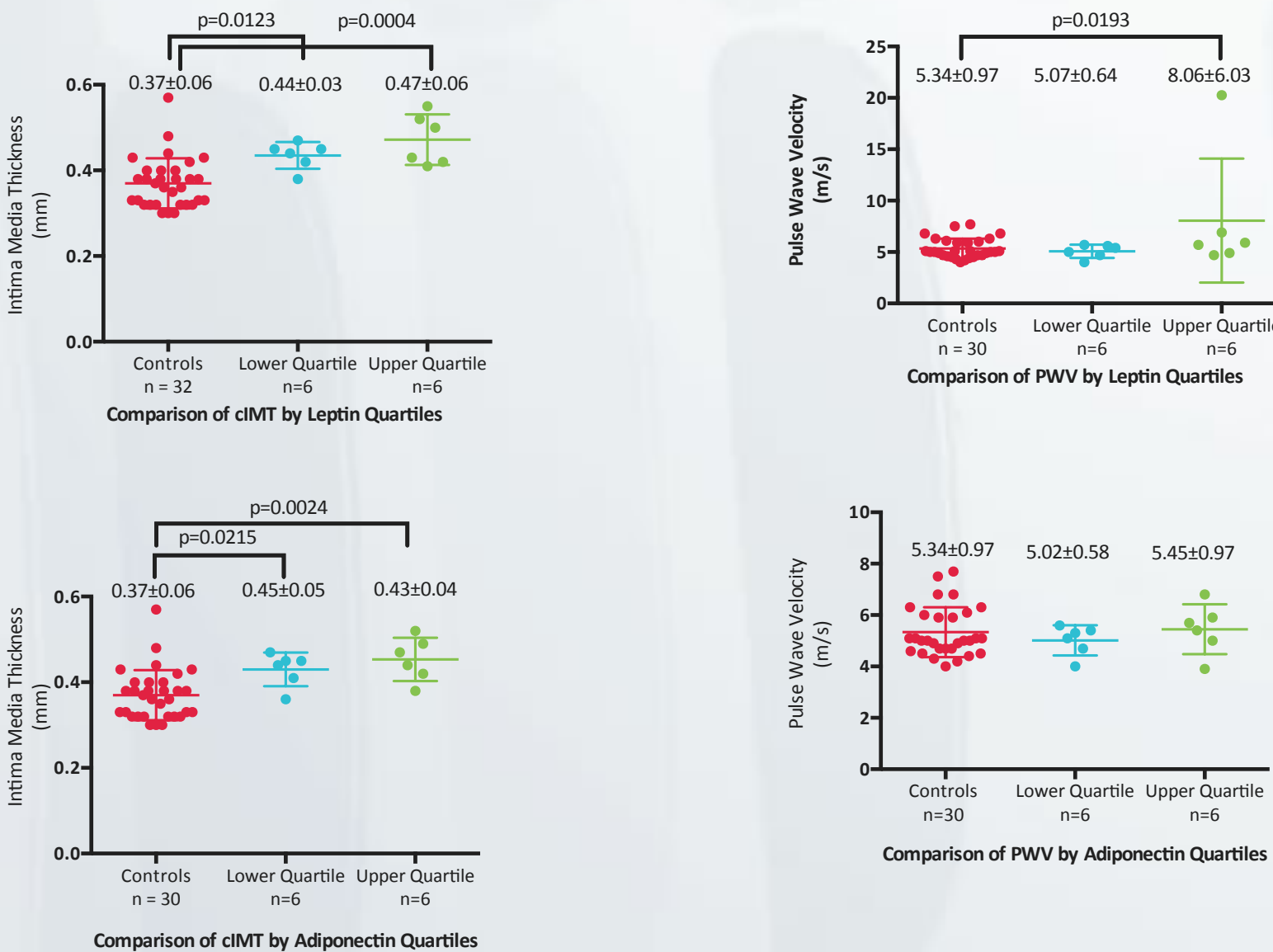


Leptin levels were 16.52(8.27 – 27.27)ng/ml in the JSLE group and 7.56(0.99-16.7)ng/ml in the control group, the difference between the medians was significant ( $p=0.0238$ ). Significant correlations were found between leptin levels and systolic BP ( $r^2=0.482$ ,  $p=0.0172$ ), PWV ( $r^2=0.433$ ,  $p=0.039$ ), serum LDL ( $r^2=0.585$ ,  $p=0.0137$ ) and BMI centiles ( $r^2=0.540$ ,  $p=0.0078$ ) in the JSLE group. Comparing leptin levels by renal BILAG score showed a tendency towards increased levels in those with active renal disease (A/B/C) but this did not reach statistical significance, see figure 34. However, those with an elevated UaUc ( $>20\text{mg}/\text{mmol}$ ) had a corresponding rise in their serum leptin levels,  $35.5\pm 38.7\text{ng}/\text{ml}$  when compared to those with low to normal UaUc ( $<20\text{mg}/\text{mmol}$ )  $18.79\pm 13.3\text{ng}/\text{ml}$ . Adiponectin levels were  $14.2\pm 9.5\text{ug}/\text{ml}$  in the JSLE group and  $12.4\pm 4.4\text{ug}/\text{ml}$  in the control group. The difference between the means was not significant ( $p=0.49$ ) and there was no increase with renal BILAG score. Significant correlations were found between adipokine levels and age ( $r^2=0.416$ ,  $p=0.0387$ ) and weekly sitting time ( $r^2=0.440$ ,  $p=0.0276$ ).



## Demographic Details

	Children (n=25)
Age (months)	170 $\pm$ 27.7
Gender (♀:♂)	22:3
eGFR (ml/min/1.73m <sup>2</sup> )	99.6 (89.9-113.9)
Disease duration (months)	33 (20.8-67.3)
Age at diagnosis (months)	123 $\pm$ 43
Cigarette smoking	0
Oral contraceptive pill	1
Positive family history of CVD	8
METS	1499 (526-3087)
Sitting minutes/day	540 (480-840)
Mean cIMT (mm)	0.4588 $\pm$ 0.04875
Mean PWV (m/s)	5.287 $\pm$ 0.727
hsCRP	0.25 (0.1-0.67)
Cholesterol (mmol/l)	5.262 $\pm$ 1.476
Triglycerides (mmol/l)	1.495 $\pm$ 1.082



## Conclusion

Adiponectin may have both anti-inflammatory effects and pro-inflammatory effects. In contrast to published data we found an increase in both leptin and adiponectin between controls and patients. Given the role played by adiponectin in protecting endothelial nitric oxide mediated vasodilation we speculate that higher serum adiponectin levels early in the JSLE phenotype could be an initial protective mechanism and could explain the increase in cIMT without an increase in PWV. It could also be indicative of a pro-inflammatory adiponectin phenotype. Our findings are in agreement with adult and paediatric studies and the relationship between serum adipokines and cIMT suggests that leptin could be used as a novel biomarker for CV risk in JSLE.



# Cardiovascular Morbidity in Juvenile-onset Systemic Lupus Erythematosus

Quinlan C, Kari J, Marks S, Tullus K.



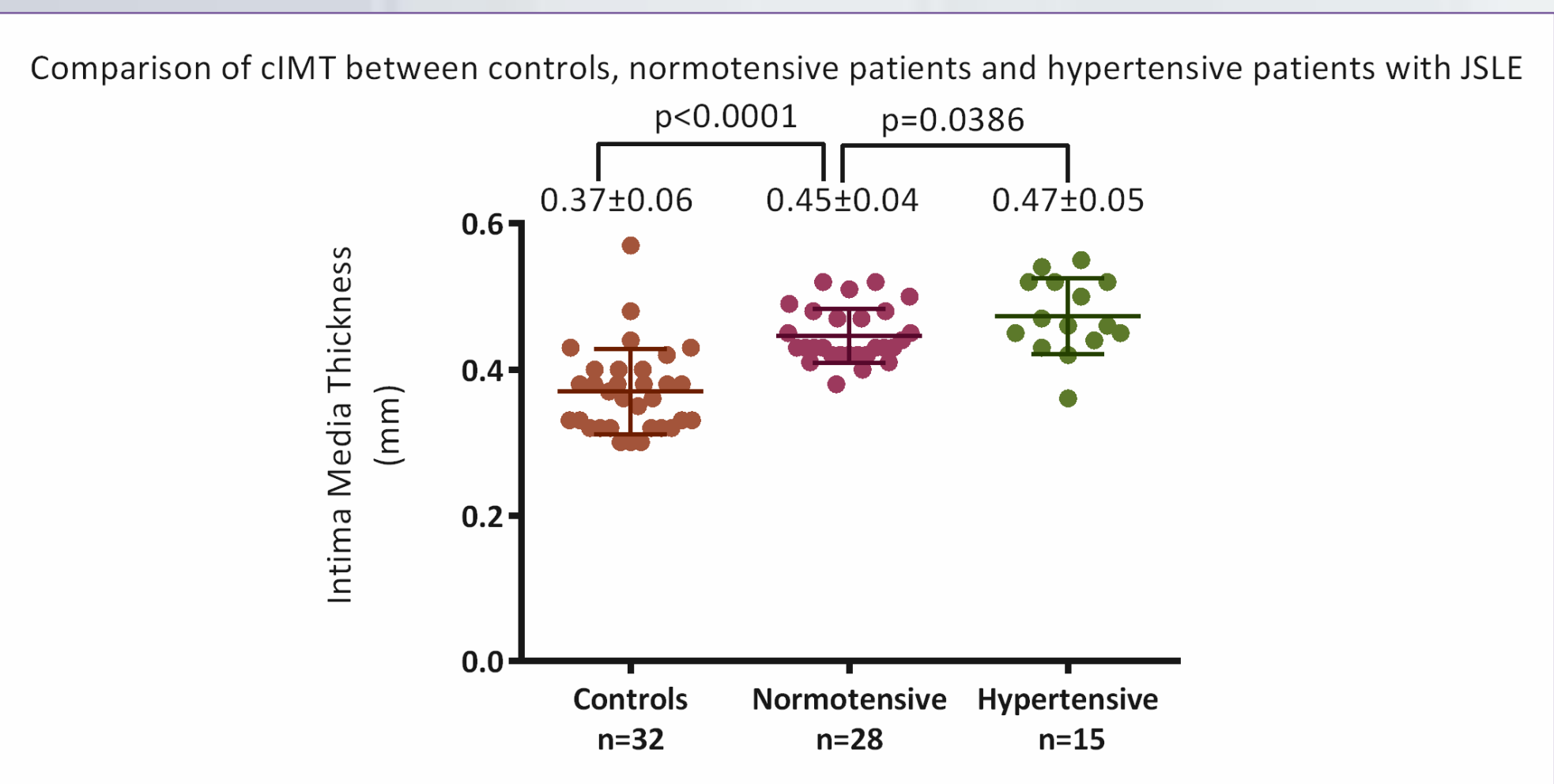
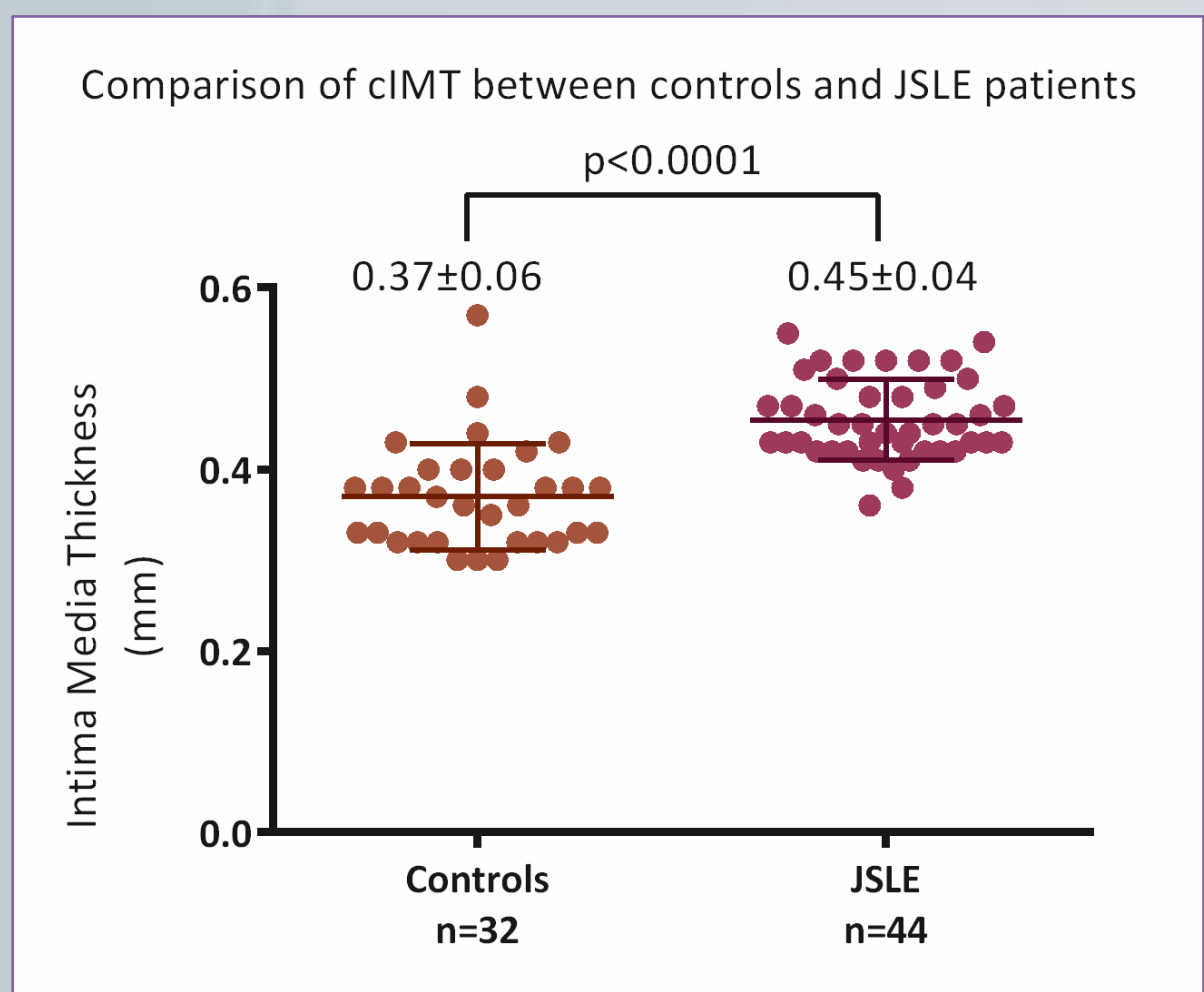
The Royal Children's Hospital, Melbourne, Australia  
Great Ormond Street Hospital for Children NHS Trust, United Kingdom

## Hypotheses

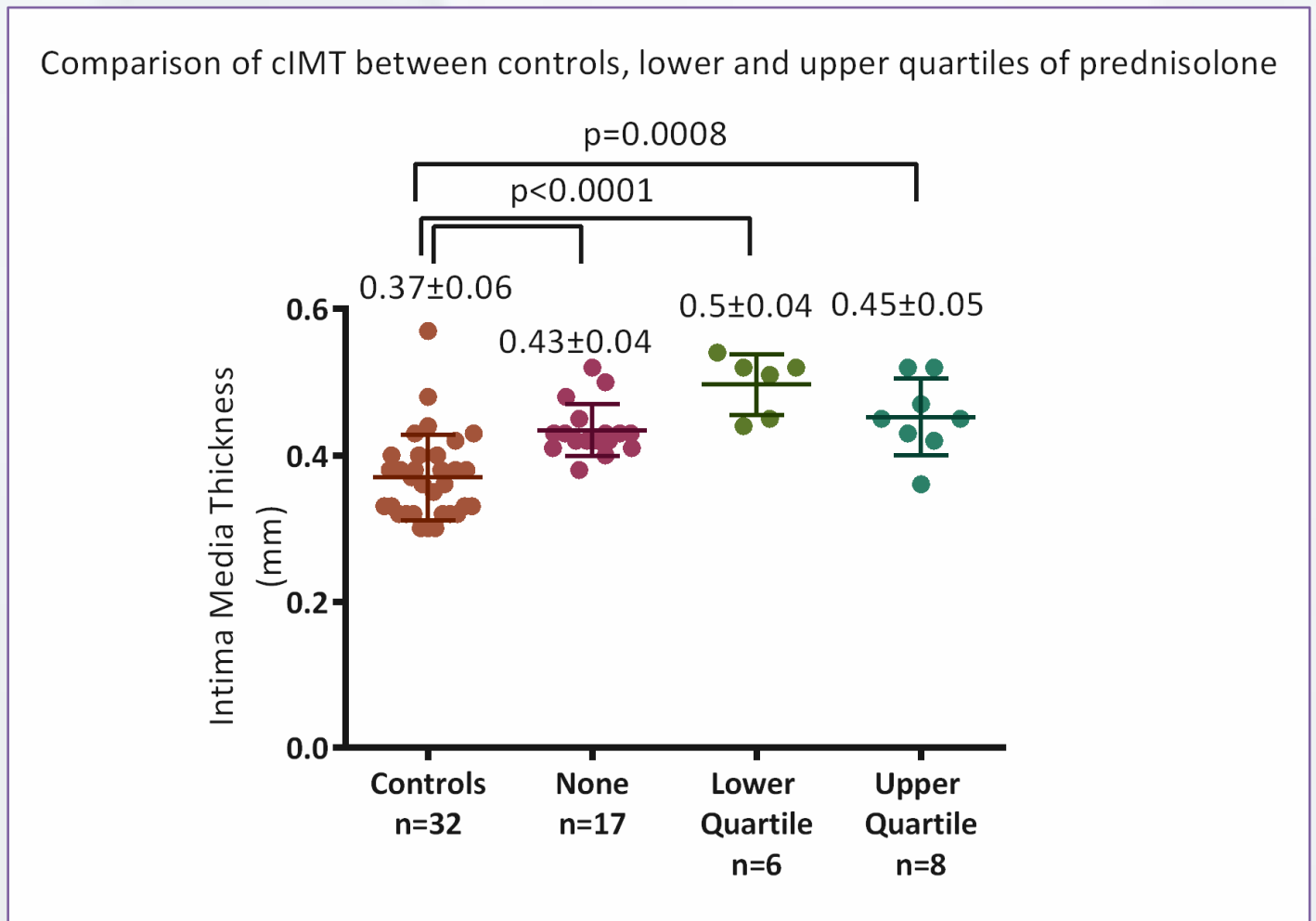
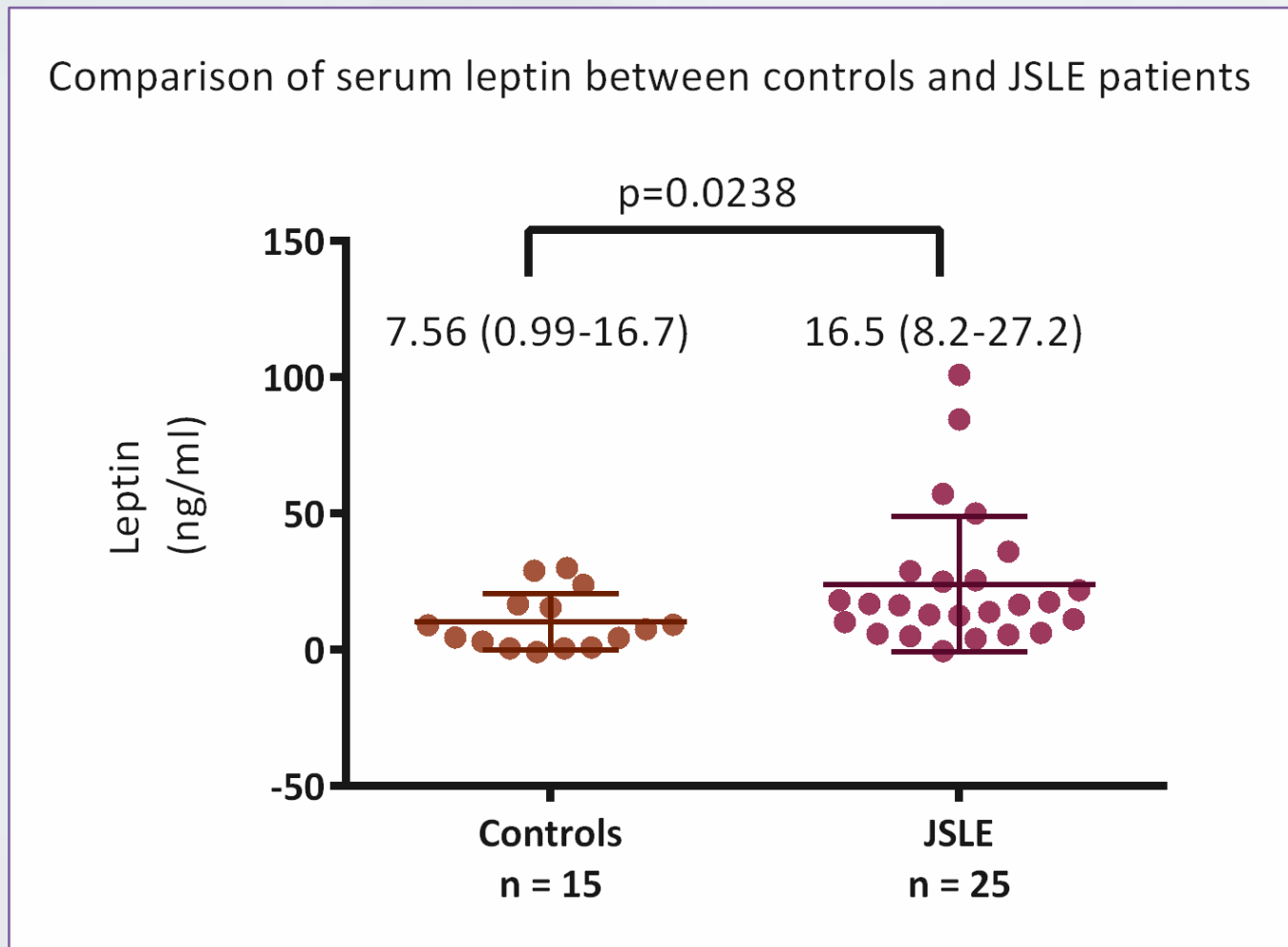
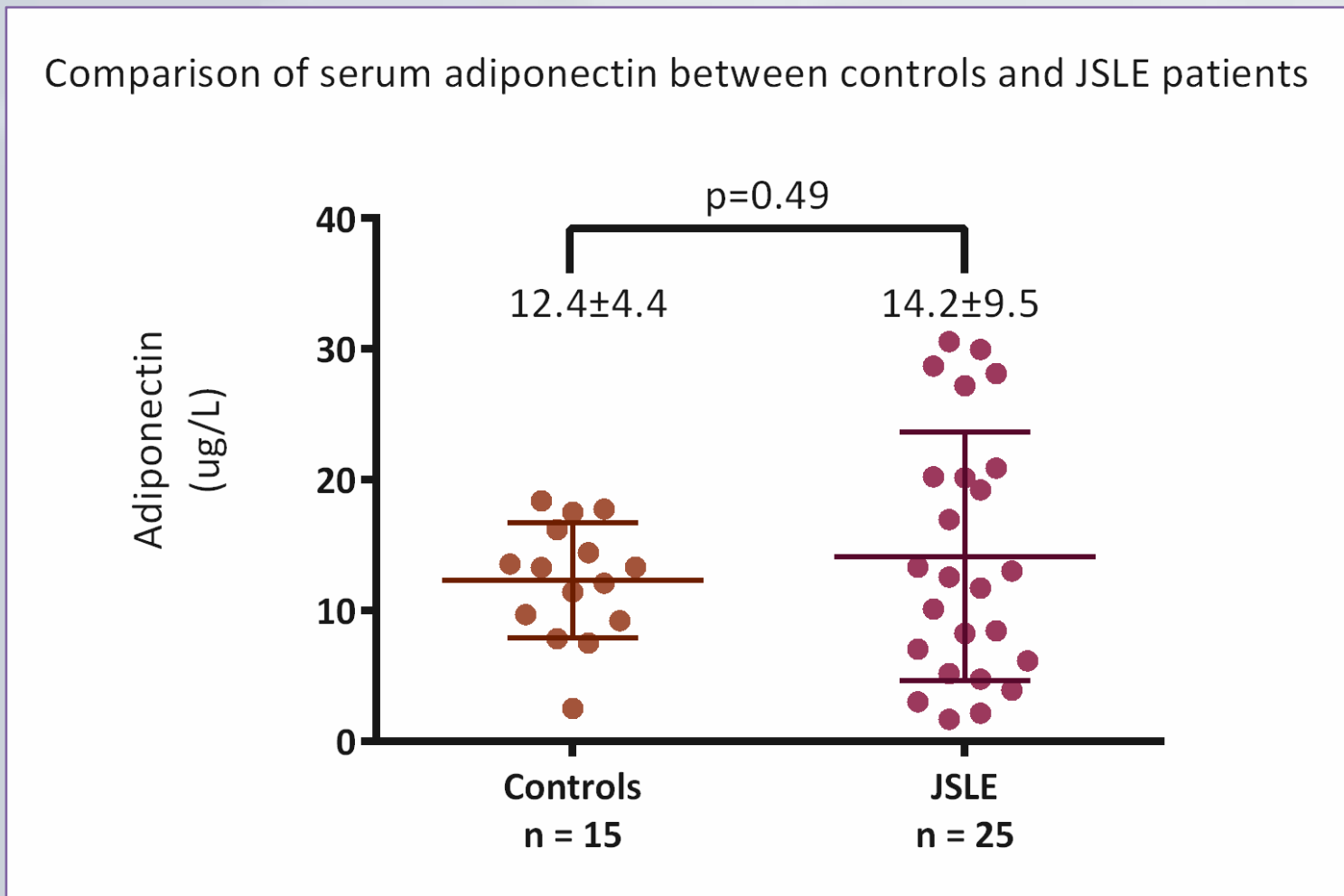
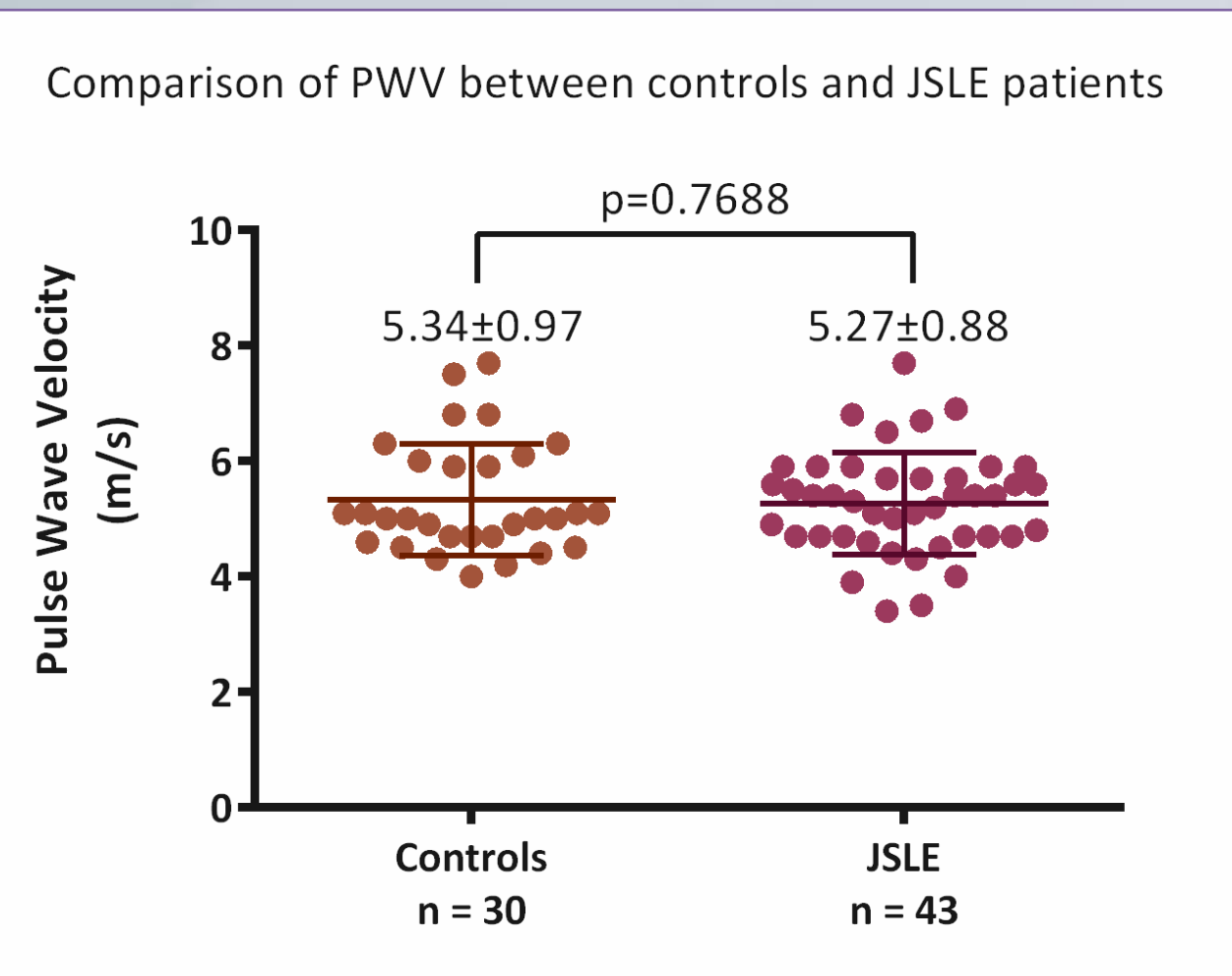
- JSLE is associated with an increased risk of CVD and that this is associated with an abnormal vascular phenotype.
- Children with JSLE have a low prevalence of the classical risk factors for CVD and thus represent a “clean” population in which to study the effect of SLE and the treatment of SLE on the vascular phenotype.
- The increased risk of CVD in JSLE is related to increased disease activity leading to abnormalities in serum lipids and adipokine activity followed by the development of an abnormal vascular phenotype.
- The increased risk of CVD in JSLE is further exacerbated by the presence of hypertension, renal impairment, the use of corticosteroids and the duration of disease.

## Study Design

- Assessment of vascular phenotype
- Evaluation of clinical and laboratory characteristics/data from routine care
- Novel biomarkers to evaluate ongoing disease and inflammation



Children with JSLE (n=45)	
Age (years)	13.5±3
Gender (♀:♂)	39:6
Weight (kg)	50.5(36.5-62)
Height (cm)	15.1±16
eGFR (ml/min/1.73m <sup>2</sup> )	103.3(93.8-113.2)
BMI	21.63(18.19-24.57)
BMI centile	65.63±28.8
Disease duration (months)	39(20.5-64.5)
Age at diagnosis (months)	115 ±42
Cigarette smoking	0
Oral contraceptive pill	1
Positive family history of CVD	10
Current Prednisolone mg/kg	0.07(0-0.23)
METS/week	1773(676.5-2854)
Sitting minutes/day	505±144.2
Mean cIMT (mm)	0.445(0.42-0.49)
Mean PWV (m/s)	5.272 ±0.88
hsCRP	0.57(0.29-1.6)
Cholesterol (mmol/l)	4.4(3.95-5.2)
Triglycerides (mmol/l)	1.04(0.76-1.93)
HDL (mmol/l)	1.28±0.46
VLDL (mmol/l)	0.45(0.33-0.83)
LDL (mmol/l)	2.35(2.14-2.99)
ESR	30.7±26.09
UAUC	2.95(1.15-55.33)



## Results

- Children with JSLE had a 23% higher mean cIMT ( $p<0.0001$ ),  $0.37 \pm 0.06$  V  $0.45 \pm 0.04$  mm
- There was a significant association between the presence of HTN and an increase in IMT, whether it was treated or not. In the non-HTN group cIMT was  $0.45 \pm 0.04$  mm and in the HTN group it was  $0.47 \pm 0.05$  mm,  $p=0.0374$
- There was a significant difference in cIMT between patients divided into upper and lower quartiles of prednisolone dose
- PWV did not differ between patients and controls  $5.27 \pm 0.88$  V  $5.34 \pm 0.97$  m/s,  $p=0.77$
- Leptin levels were  $16.52(8.27 - 27.27)$  ng/ml in the JSLE group and  $7.56(0.99-16.7)$  ng/ml in the control group,  $p=0.0238$
- cIMT increased with increasing leptin levels when analysed by quartiles. The lower leptin quartile group had a cIMT of  $0.44 \pm 0.03$  mm increasing to  $0.47 \pm 0.06$  mm in the higher quartile group,  $p=0.0004$
- Adiponectin levels were  $14.2 \pm 9.5$  ug/ml in the JSLE group and  $12.4 \pm 4.4$  ug/ml in the control group. The difference between the means was not significant ( $p=0.49$ ) and there was no increase with renal pBILAG score.

## Conclusion

- There is a low prevalence of traditional CV risk factors in patients with JSLE
- Patients had evidence of structural changes in blood vessels with possible functional adaptation
- There was an increase in serum leptin and adiponectin levels which was associated with increased cIMT
- There was no clear evidence of increased CV risk in patients with JSLE at this stage



# Appendices



## **Appendix 1: Ethics approval for CVJSLE study**



10. No. 74



## National Research Ethics Service

NRES Committee London - Dulwich

(formerly South East London REC 3, formerly King's College Hospital Research Ethics Committee)  
1st Floor Camberwell Building  
King's College Hospital  
94 Denmark Hill  
London  
SE5 9RS

22 JUN 2011

Telephone: 020 3299 3923  
Facsimile: 020 3299 5085

17 June 2011

Dr Kjell Tullus  
Consultant Paediatric Nephrologist  
Great Ormond Street Hospital for Children  
Great Ormond Street  
WC1N 3JH

Dear Dr Tullus

**Study title:** Cardiovascular morbidity in children and young people  
with systemic lupus erythematosus - prevalence and  
risk factors  
**REC reference:** 11/LO/0555

Thank you for your email correspondence of 15 June 2011, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

### Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

### Ethical review of research sites

#### NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

#### Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

### Conditions of the favourable opinion

*The favourable opinion is subject to the following conditions being met prior to the start of the study.*

*Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.*

*Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.*

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>.

*Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.*

*For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.*

*Sponsors are not required to notify the Committee of approvals from host organisations*

**It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).**

#### **Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Covering Letter		
GP/Consultant Information Sheets	1 (CP)	01 June 2011
GP/Consultant Information Sheets	1 (KT)	01 June 2011
Investigator CV		
Email correspondence from Dr Kjell Tullus		15 June 2011
Participant Consent Form: Control	1	01 June 2011
Participant Consent Form: Control	2	01 June 2011
Participant Consent Form: Parents/Guardians - Control	1	01 June 2011
Participant Consent Form: Parents/Guardians - Control	2	01 June 2011
Participant Consent Form: Assent form	1	01 June 2011
Participant Consent Form: Assent form	2	01 June 2011
Participant Information Sheet: Parent/Guardian	1	04 June 2010
Participant Information Sheet: Patient	1	04 June 2010
Participant Information Sheet: Control	1	01 June 2011
Participant Information Sheet: Parents/Guardians	2	01 June 2011
Participant Information Sheet: Patients	2	01 June 2011
Participant Information Sheet: Patients (12-15 years)	2	01 June 2011
Participant Information Sheet: Parents/Guardians (Controls)	1	01 June 2011
Participant Information Sheet: Control (12-15 years)	1	01 June 2011
Protocol	2	11 April 2011
REC application		11 April 2011



## Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

## After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "*After ethical review – guidance for researchers*" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email [referencegroup@nres.npsa.nhs.uk](mailto:referencegroup@nres.npsa.nhs.uk).

11/LO/0555

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely



Dr David Jewitt  
Chair

Email: [juliet.kirk-buaku@nhs.net](mailto:juliet.kirk-buaku@nhs.net)

Enclosures: "After ethical review – guidance for researchers" [SL-AR2]

Copy to: Mrs Gill Lambert, UCL Institute of Child Health  
Ms Nima Sharma, Great Ormond Street Hospital for Children NHS Trust





## Appendix 2: Vascular physiology certification



# *Certificate of Accreditation*

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This is to certify that

**CATHY QUINLAN**

---

Has successfully completed training and accreditation in cIMT and PWV scanning



**UCL, Institute of Child  
Health**

Accredited By: Devina Bhowruth    16.11.2011

Prof. John Deanfield : Vascular Physiology Unit, Institute Of Child Health

### **Appendix 3: GOSH information sheets and consents**



# Great Ormond Street

## Hospital for Children

NHS Trust

Great Ormond Street  
London WC1N 3JH

Tel: 020 7405 9200

### **Information for PARENTS / GUARDIANS of young people who are patients at Great Ormond Street Hospital & UCLH Adolescent Unit**

#### Cardio-Vascular risk in children and young people with Systemic Lupus Erythematosus (SLE)

We would like to ask for you and your child to help us with this study on the optimal management of the heart and blood vessel changes in patients with SLE.

#### **Why is the study being done?**

We know that young adults with SLE have an increased risk of heart attack and stroke compared to their unaffected peers. We are trying to identify those most at risk earlier in life to help minimise the long term problems.

The researchers believe this study will lead to the development of guidelines for the correct investigations and thus optimal management of the patient's cardio-vascular health as they grow. We want to do that by gathering information about how blood vessels are affected by SLE and what causes those changes.

#### **How is the study to be done?**

Your child will be in the study for a minimum of two years and a maximum of five years to monitor their disease progress. We want to monitor the effects of the disease and its treatment on their cardio-vascular health.

The treatment of your child will not be influenced by the investigations in the study. This will, as always, be decided by how your child is doing in him or herself and the results of the routine blood tests.

The extra blood samples will be taken with your child's routine blood tests. The physiological investigations may possibly need to be done at an alternative time which we will ensure is convenient for you. You will be reimbursed for the travel costs on these occasions.

The results from the lupus children will be compared to those of a control group made up of children with diseases that should not have any influence on the studied parameters; from other clinics at GOSH.

This control group will be of children similar in age, sex and ethnicity to the study group and be approximately the same size to ensure accurate results

We will collect a urine sample and an extra 10mls of blood with routine tests. The blood and the urine will be analysed for different blood substances and cells. We will also look for evidence of blood vessel damage.

We will ask your permission to use some of the blood taken for ethically approved research such as, (but not exclusively) tests for analysing cardio-vascular health / medication effectiveness / genetic causes of SLE; which may be inherited by their children.

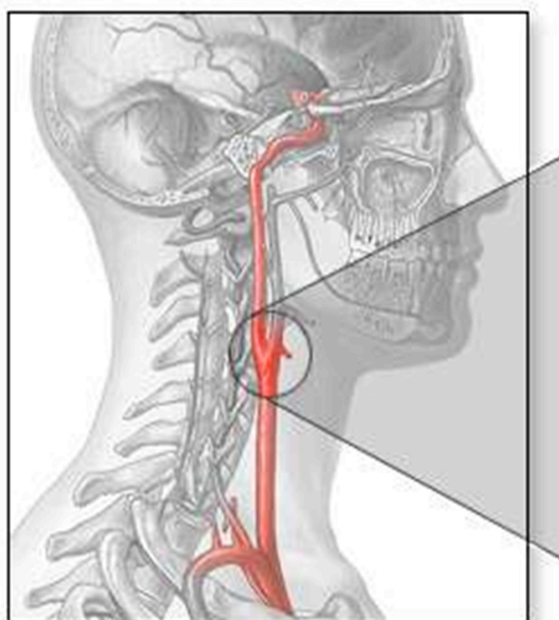
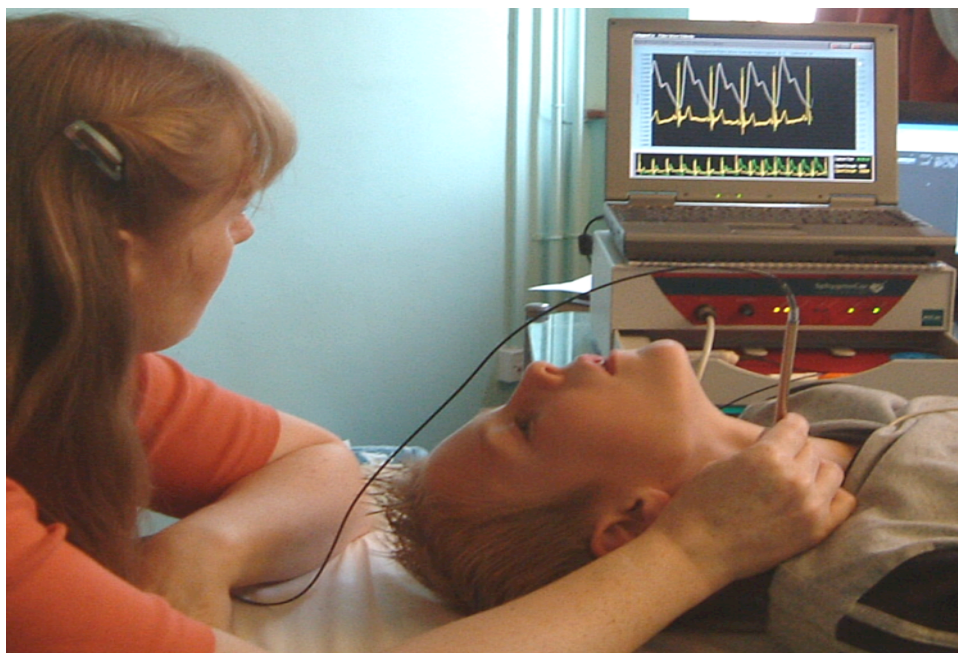
We will study the blood vessels and blood flow in two ways using ultra-sound, when they enter the study and again if they have any relapses while on routine treatment.

- 1) Measure thickness of the artery walls in the neck
- 2) Measure how quickly blood travels down the arms and legs

**Are there risks and discomforts?**

There is of course a small risk that we will discover something abnormal during the research investigations that we did not previously know about your child; we will of course discuss these findings with you.

Your child will not need any extra blood tests. The tests to examine the blood vessels with ultra-sound are painless.





**What are the potential benefits?**

As your child will be receiving additional investigations looking specifically at risk factors during the study we may be able to make changes to their treatment and thus minimise future problems.

**Who will have access to my child's case notes and research records?**

*The information gathered will be stored in a secure data base using their JSLE registration number; accessible only to your usual doctors, nurses, members of the research team and representatives of the Research Ethics Committee.*

**Do we have to take part in this study?**

If you decide that you do not wish your child to participate in this research project, which is entirely your right, it will not in any way prejudice any present or future treatment. If they do enter the study and then wish to withdraw, at a later date, this still applies but we will keep all anonymised data collected until that time.

**Who can we ask about this study?**

You can ask your family, the doctors and nurses you usually see in clinic; you can also discuss anything you may need further information about with Catherine the assistant researcher - 0207 905 2695

**What are the arrangements for compensation, should any harm come?**

An independent research ethics committee who believe that it is of minimal risk to your child has approved this project. However, research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm should occur as a result of taking part in this study. No special compensation arrangements have been made for this project but you have the right to claim damages in a court of law. This would require you to prove fault on the part of the hospital or any manufacturer involved.

**Who do we speak to if problems arise?**

If you have any complaints about the way in which this research project has been, or is being conducted, please discuss them with the researchers. If the problems are not resolved, or you wish to comment in any other way, please contact – PALS via the hospital switchboard 0207 405 9200

**Information for your GP**

We will write to your GP to inform them of your child's participation in this study and inform them of any relevant results via the GP clinic letter.

**Researchers who will have contact with the family**

*Dr Kjell Tullus*

*Consultant Paediatric Nephrologist  
0207 405 9200 ext 0292*

*Dr Clarissa Pilkington*

*Consultant Paediatric Rheumatologist  
0207 405 9200 ext 5334*

# Great Ormond Street

## Hospital for Children

NHS Trust

Great Ormond Street  
London WC1N 3JH

Tel: 020 7405 9200

### **Information for PATIENTS attending Great Ormond Street Hospital & UCLH Adolescent Unit**

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We would like to ask for your help with this study on the optimal management of heart and blood vessel changes in patients with SLE.

#### **Why is the study being done?**

We know that young adults with SLE have an increased risk of heart attack and stroke compared to their unaffected peers. We are trying to identify those at most risk earlier in life to help minimise the long term problems.

The researchers believe this study will lead to the development of guidelines for the correct investigations and thus optimal management of the patient's cardio-vascular health as they grow. We want to do that by gathering information about how blood vessels are affected by SLE and what causes those changes.

#### **How is the study to be done?**

You will be in the study for a minimum of two years and a maximum of five years to monitor your disease progress. We want to monitor the effects of the disease and its treatment on your cardio-vascular health.

Your treatment will not be influenced by the investigations in the study. This will, as always, be decided by how you are doing in yourself and the results of the routine blood tests.

The blood samples will be taken with your regular blood tests. The physiological investigations may possibly need to be done at an alternative time which we will ensure is convenient for you. You will be reimbursed for the travel costs on these occasions.

The results from the lupus children will be compared to those of a control group made up of children with diseases that should not have any influence on the studied parameters; from other clinics at GOSH.

This control group will be of children similar in age, sex and ethnicity to the study group and be approximately the same size to ensure accurate results

We will collect a urine sample and an extra 10mls of blood with routine tests. The blood and the urine will be analysed for different blood substances and cells. We will also look for evidence of blood vessel damage.

We will ask your permission to use the blood taken for ethically approved research such as, (but not exclusively) tests for analysing cardio-vascular health / medication effectiveness / genetic causes of SLE; which may be inherited by your children.

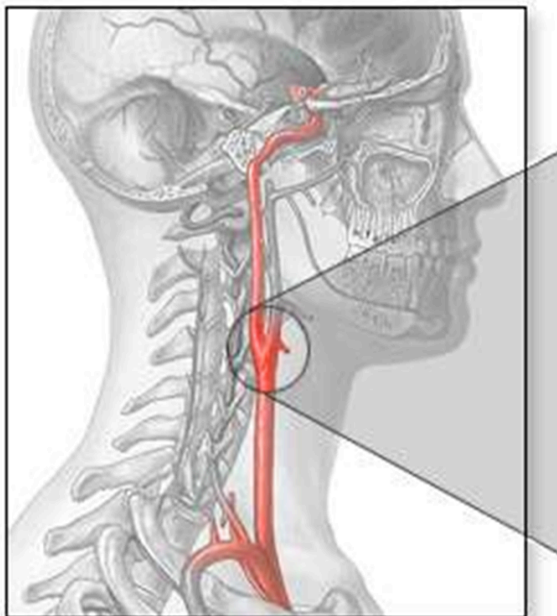
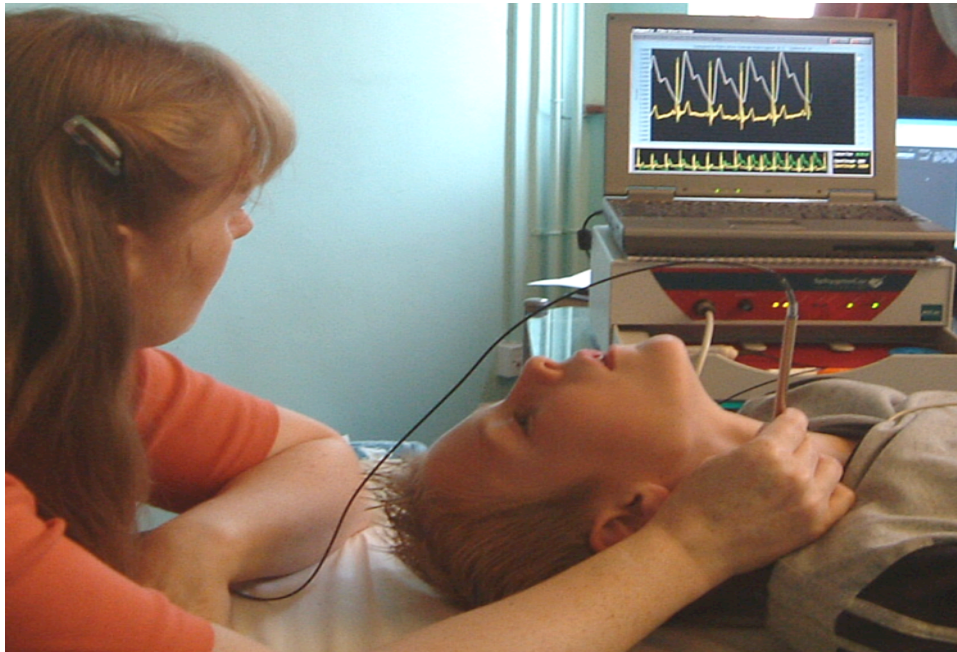
We will study the blood vessels and blood flow in two ways using ultra-sound, when you enter the study and again if you have any relapses while on routine treatment.

- 1) Measure thickness of the artery walls in the neck.
- 2) Measure how quickly blood travels down the arms and legs.

**Are there risks and discomforts?**

There is of course a small risk that we will discover something abnormal during the research investigations that we did not previously know about you; we will of course discuss these findings with you.

You will not need any extra blood tests. The tests to examine the blood vessels with ultra-sound are painless and the test to see response of the vessels to occluding blood flow causes the same amount of discomfort as the electronic BP machine



**What are the potential benefits?**

As you will be receiving additional investigations looking specifically at risk factors during the study we may be able to make changes to your treatment and thus minimise future problems.

**Who will have access to my case notes and research records?**

The information gathered will be stored in a secure data base using your JSLE registration number; accessible only to your usual doctors, nurses, members of the research team and representatives of the Research Ethics Committee.

**Do I have to take part in this study?**

If you decide that you do not wish to participate in this research project, which is entirely your right, it will not in any way prejudice any present or future treatment. If you do enter the study and then wish to withdraw, at a later date, this still applies but we will keep all anonymised data collected until that time.

**Who can I ask about this study?**

You can ask your family, the doctors and nurses you usually see in clinic; you can also discuss anything you may need further information about with Catherine who is the assistant researcher - 0207 905 2695

**What are the arrangements for compensation, should any harm come?**

An independent research ethics committee who believe that it is of minimal risk to you has approved this project. However, research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm should occur as a result of taking part in this study. No special compensation arrangements have been made for this project but you have the right to claim damages in a court of law. This would require you to prove fault on the part of the hospital or any manufacturer involved.

**Who do I speak to if problems arise?**

If you have any complaints about the way in which this research project has been, or is being conducted, please discuss them with the researchers. If the problems are not resolved, or you wish to comment in any other way, please contact – PALS via the hospital switchboard 0207 405 9200.

**Information for your GP**

We will write to your GP to inform them of your participation in this study and inform them of any relevant results via the GP clinic letter.

**Researchers who will have contact with the family**

*Dr Kjell Tullus*  
*Consultant Paediatric Nephrologist*  
*0207 405 9200 ext 0292*

*Dr Clarissa Pilkington*  
*Consultant Paediatric Rheumatologist*  
*0207 405 9200 ext 5334*

# Great Ormond Street

## Hospital for Children

NHS Trust

Great Ormond Street  
London WC1N 3JH

Tel: 020 7405 9200

### **Information for parents and guardians of young people who are being investigated as CONTROL SUBJECTS in a cardio-vascular study.**

#### Cardio-Vascular risk in children and young people with Systemic Lupus Erythematosus (SLE)

We would like to ask for you and your child to help us with this study on the optimal management of the heart and blood vessel changes in patients with SLE; by your child being investigated as a control subject so we can look at the differences between young people with SLE and young people without SLE.

#### **Why is the study being done?**

We know that young adults with SLE have an increased risk of heart attack and stroke compared to their unaffected peers. We are trying to identify those at most risk earlier in life to help minimise the long term problems.

We want to do that by gathering information about how blood vessels are affected by SLE and what causes those changes. To ensure accurate results we need to investigate a control group of young people without SLE.

#### **How is the study to be done?**

We will collect a urine sample and 10mls (two teaspoons) of blood, which will be analysed for known indicators of blood vessel damage.

We will ask your permission to use some of the blood taken for ethically approved research such as tests for analysing cardio-vascular health in children without SLE.

The extra blood samples will be taken with your child's routine blood tests, if your child does not have routine blood tests they will have to agree with having these done to take part in the study; neither you nor the researchers can force your child to have a blood test against their will.

The physiological investigations may possibly need to be done at an alternative time which we will ensure is convenient for you. You will be reimbursed for the travel costs on these occasions.

We will study the blood vessels and blood flow in three ways using ultra-sound –

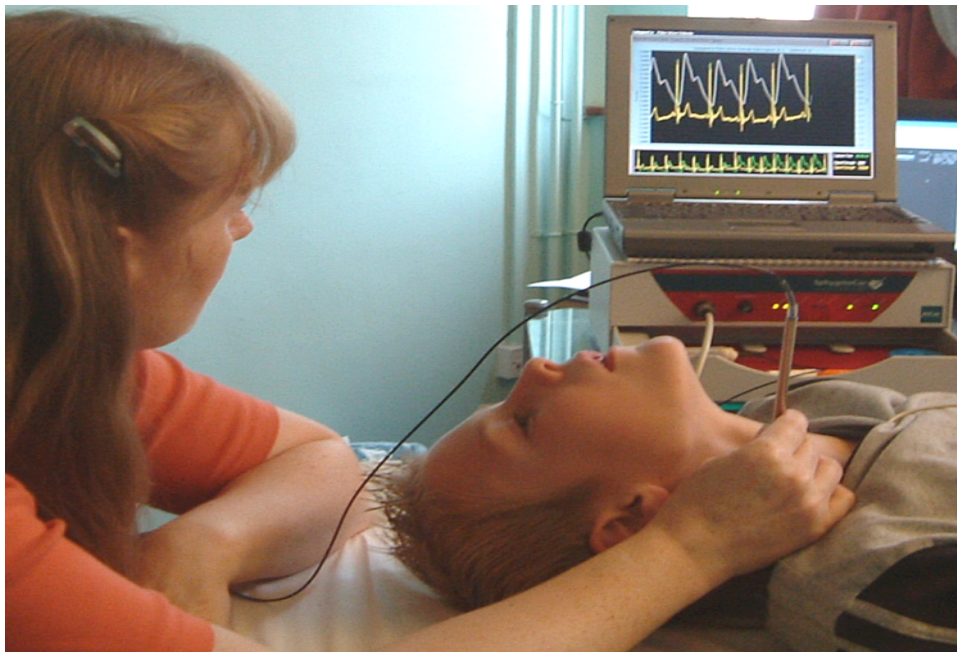
- 1) Measure thickness of the artery walls in the neck
- 2) Measure how quickly blood travels down the arms and legs
- 3) Measure the effect of occluding the blood flow down the arms using a BP cuff and how the blood vessels respond to this

### **Are there risks and discomforts?**

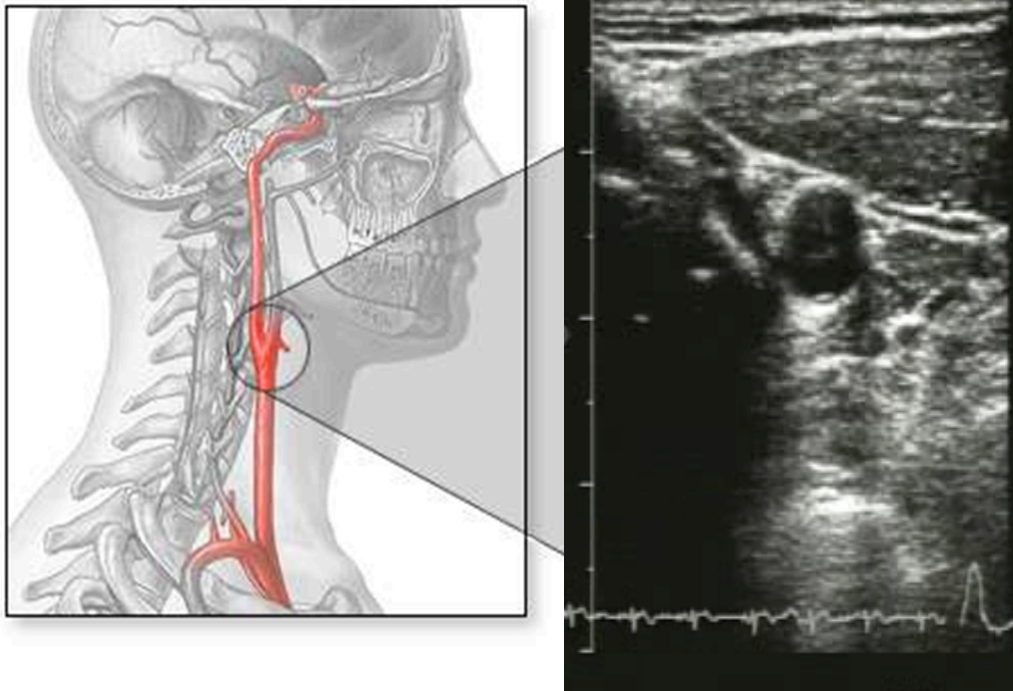
There is of course a small risk that we will discover something abnormal during the research investigation that was previously unknown about your child; we will of course discuss these findings with you and your child's consultant.

There is a risk of pain and bruising at the blood test site, but this will be minimised by local anaesthetic agents and the sample will be taken by an expert practitioner.

The tests to examine the blood vessels with ultra-sound are painless and the test to see response of the vessels to occluding blood flow causes the same discomfort as the electronic Blood Pressure machine, if your child has no experience of the electronic BP machine we will offer them a chance to experience what it feels like prior to doing that part of the investigations.







**What are the potential benefits?**

There are no expected benefits for your child but obviously there is a chance we may discover something that needs further investigation and we will refer your child to the appropriate medical team if this occurs.

**Do we have to take part in this study?**

If you decide that you do not wish your child to participate in this research project, which is entirely your right, it will not in any way prejudice any present or future treatment. If they do enter the study and then wish to withdraw, at a later date, this still applies but we will keep all anonymised data collected until that time.

**Who can we ask about this study?**

You can ask your family, the doctors and nurses you usually see in clinic; you can also discuss anything you may need further information about with Ambrose and Cathy who are the assistant researchers - 0207 905 2695

**Who will have access to my child's case notes and research records?**

The information gathered will be stored in a secure data base using their hospital registration number; accessible only to your usual doctors, nurses, members of the research team and representatives of the Research Ethics Committee.



### **What are the arrangements for compensation, should any harm come?**

An independent research ethics committee who believe that it is of minimal risk to your child has approved this project. However, research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm should occur as a result of taking part in this study. No special compensation arrangements have been made for this project but you have the right to claim damages in a court of law. This would require you to prove fault on the part of the hospital or any manufacturer involved.

### **Who do we speak to if problems arise?**

If you have any complaints about the way in which this research project has been, or is being conducted, please discuss them with the researchers. If the problems are not resolved, or you wish to comment in any other way, please contact – PALS via the hospital switchboard 0207 405 9200

### **Information for your GP**

We will write to your GP to inform them of your child's participation in this study and any relevant results that need further investigation.

### **Researchers who will have contact with the family**

Dr Kjell Tullus  
Consultant Paediatric Nephrologist  
0207 405 9200 ext 0292

Dr Clarissa Pilkington  
Consultant Paediatric Rheumatologist  
0207 405 9200 ext 5334

# Great Ormond Street

## Hospital for Children

NHS Trust

Great Ormond Street  
London WC1N 3JH

Tel: 020 7405 9200

### **Information for young people who are being investigated as CONTROL SUBJECTS in a cardio-vascular study.**

#### Cardio-Vascular risk in children and young people with Systemic Lupus Erythematosus (SLE)

We would like to ask for your help with this study on the optimal management of the heart and blood vessel changes in patients with SLE; by being investigated as a control subject, so we can look at the differences between young people with SLE and young people without SLE.

#### **Why is the study being done?**

We know that young adults with SLE have an increased risk of heart attack and stroke compared to their unaffected peers. We are trying to identify those at most risk earlier in life to help minimise the long term problems.

We want to do that by gathering information about how blood vessels are affected by SLE and what causes those changes. To ensure accurate results we need to investigate a control group of young people without SLE.

#### **How is the study to be done?**

We will collect a urine sample and 10mls (two teaspoons) of blood, which will be analysed for known indicators of blood vessel damage.

We will ask your permission to use some of the blood taken for ethically approved research such as tests for analysing cardio-vascular health in children without SLE.

The extra blood samples will be taken with your routine blood tests, if you do not normally have blood tests, you will have to agree with having these to take part in the study; your parents or the researchers cannot force you to have a blood test against your will.

The physiological investigations may possibly need to be done at an alternative time which we will ensure is convenient for you. You will be reimbursed for the travel costs on these occasions.

We will study the blood vessels and blood flow in three ways using ultra-sound –

- 1) Measure thickness of the artery walls in the neck

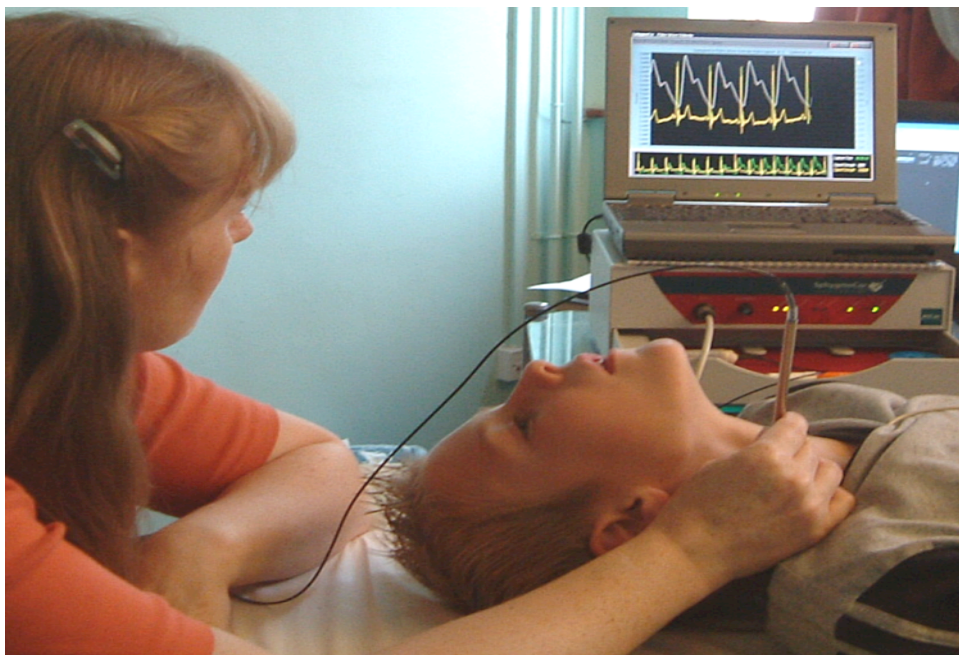
- 2) Measure how quickly blood travels down the arms and legs
- 3) Measure the effect of occluding the blood flow down the arms using a BP cuff and how the blood vessels respond to this

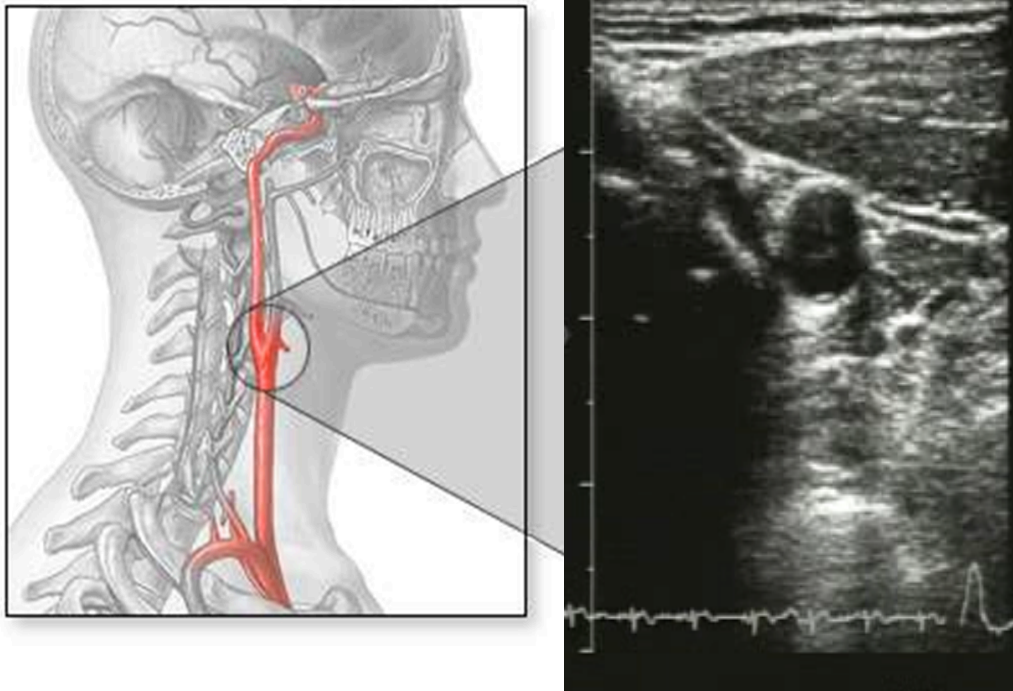
### **Are there risks and discomforts?**

There is of course a small risk that we will discover something abnormal during the research investigation that was previously unknown about you; we will of course discuss these findings with you and your consultant.

There is a risk of pain and bruising at the blood test site, but this will be minimised by local anaesthetic agents and the sample will be taken by an expert practitioner.

The tests to examine the blood vessels with ultra-sound are painless and the test to see response of the vessels to occluding blood flow causes the same discomfort as the electronic Blood Pressure machine, if you have no experience of the electronic BP machine we will offer you a chance to experience what it feels like prior to doing that part of the investigations.





**What are the potential benefits?**

There are no expected benefits, but obviously there is a chance we may discover something that needs further investigation and we will refer you to the appropriate medical team if this occurs.

**Do I have to take part in this study?**

If you decide that you do not wish to participate in this research project, which is entirely your right, it will not in any way prejudice any present or future treatment. If you do enter the study and then wish to withdraw, at a later date, this still applies but we will keep all anonymised data collected until that time.

**Who can I ask about this study?**

You can ask your family, the doctors and nurses you usually see in clinic; you can also discuss anything you may need further information about with Ambrose and Cathy who are the assistant researchers - 0207 905 2695

**Who will have access to my case notes and research records?**

The information gathered will be stored in a secure data base using your hospital registration number; accessible only to your usual doctors, nurses, members of the research team and representatives of the Research Ethics Committee.

### **What are the arrangements for compensation, should any harm come?**

An independent research ethics committee who believe that it is of minimal risk to your child has approved this project. However, research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm should occur as a result of taking part in this study. No special compensation arrangements have been made for this project but you have the right to claim damages in a court of law. This would require you to prove fault on the part of the hospital or any manufacturer involved.

### **Who do I speak to if problems arise?**

If you have any complaints about the way in which this research project has been, or is being conducted, please discuss them with the researchers. If the problems are not resolved, or you wish to comment in any other way, please contact – PALS via the hospital switchboard 0207 405 9200

### **Information for your GP**

We will write to your GP to inform them of your participation in this study and any relevant results that need further investigation.

### **Researchers who will have contact with the family**

Dr Kjell Tullus  
Consultant Paediatric Nephrologist  
0207 405 9200 ext 0292

Dr Clarissa Pilkington  
Consultant Paediatric Rheumatologist  
0207 405 9200 ext 5334

# Great Ormond Street

## Hospital for Children

NHS Trust

Great Ormond Street  
London WC1N 3JH

Tel: 020 7405 9200

### **Information for young people age 12 – 15 yrs. who are being investigated as CONTROL SUBJECTS in a cardio-vascular study.**

#### Cardio-Vascular risk in children and young people with Systemic Lupus Erythematosus (SLE)

We would like to ask for your help with this study by being investigated as a control subject, so we can look at the differences between young people with SLE and young people without SLE.

#### **Why is the study being done?**

We are trying to identify those at most risk of heart problems earlier in life to help minimise the long term problems.

We want to do that by gathering information about how blood vessels are affected and what causes those changes.

To ensure accurate results we need to investigate a control group of young people without SLE.

#### **How is the study to be done?**

We will collect a urine sample and 10mls (two teaspoons) of blood, which we will look at for signs of blood vessel damage.

The extra blood samples will be taken with your routine blood tests, if you do not normally have blood tests, you will have to agree with having them to take part in the study; your parents or the researchers cannot force you to have a blood test against your will.

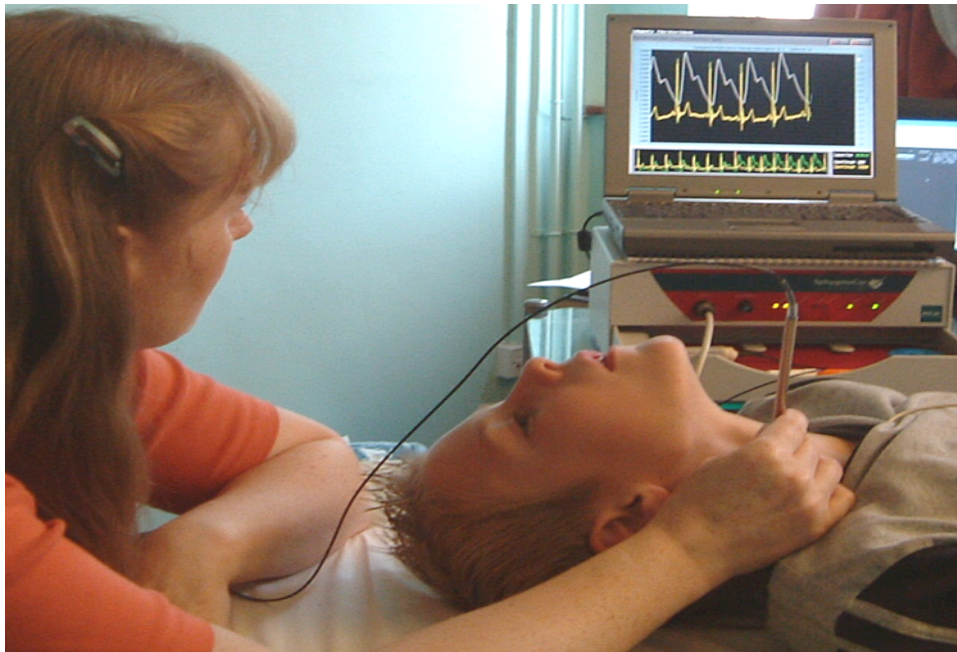
We will study the blood vessels and blood flow in three ways using ultra-sound –

- 1) Measure thickness of the artery walls in the neck
- 2) Measure how quickly blood travels down the arms and legs
- 3) Measure the effect of occluding the blood flow down the arms using a BP cuff and how the blood vessels respond to this

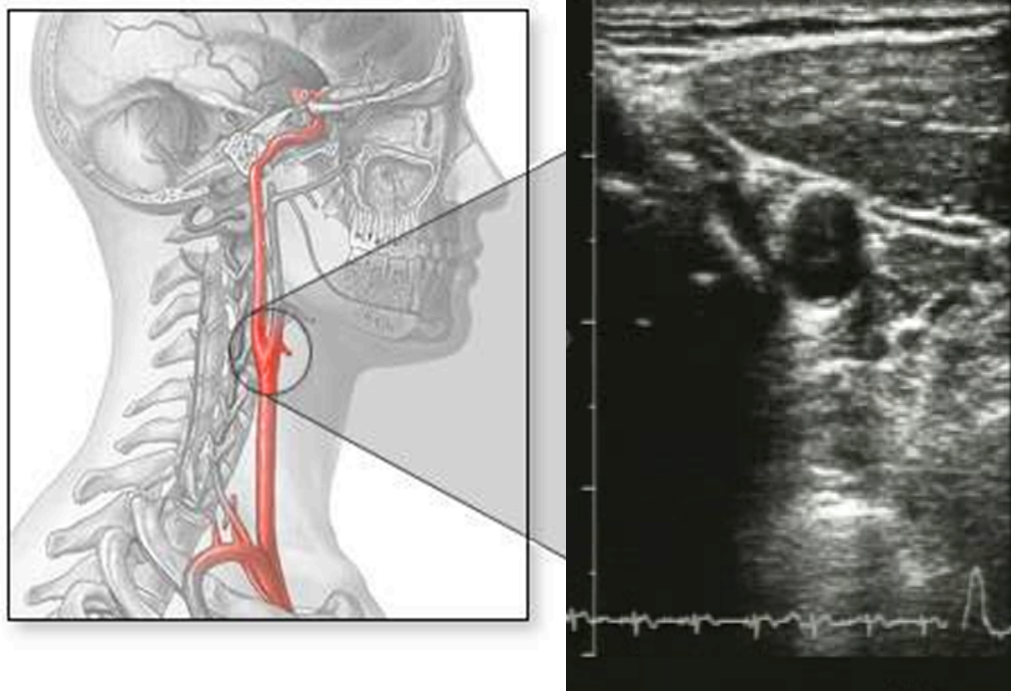
### **Are there risks and discomforts?**

There is a risk of pain and bruising at the blood test site, but this will be minimised by local anaesthetic agents and the sample will be taken by an expert practitioner.

The tests to examine the blood vessels with ultra-sound are painless and the test to see response of the vessels to occluding blood flow causes the same discomfort as the electronic Blood Pressure machine, if you have no experience of the electronic BP machine we will offer you a chance to experience what it feels like prior to doing that part of the investigations.







### **What are the potential benefits?**

There are no expected benefits, but obviously there is a chance we may discover something that needs further investigation and we will refer you to the appropriate medical team if this occurs.

### **Do I have to take part in this study?**

If you decide that you do not wish to participate in this research project, which is entirely your right, it will not change anything and if you do enter the study and then want to stop again, that is ok too.

### **Who can I ask about this study?**

You can ask your family, the doctors and nurses you usually see in clinic; you can also discuss anything you may need further information about with Ambrose and Cathy who are the assistant researchers - 0207 905 2695

### **Who will have access to my case notes and research records?**

The information gathered will be stored in a secure data base using your hospital registration number; accessible only to your usual doctors, nurses, members of the research team and representatives of the Research Ethics Committee.

### **Information for your GP**



We will write to your GP to inform them of your participation in this study and any relevant results that need further investigation.

**Researchers who will have contact with the family**

Dr Kjell Tullus  
Consultant Paediatric Nephrologist  
0207 405 9200 ext 0292

Dr Clarissa Pilkington  
Consultant Paediatric Rheumatologist  
0207 405 9200 ext 5334

**Consent Form for PARENTS / GUARDIANS  
of Children Participating in Research Studies**

Cardio-Vascular risk in children and young people with  
Systemic Lupus Erythematosus (SLE / Lupus)

**NOTES FOR PARENTS OR GUARDIANS**

1. Your child has been asked to take part in a research study. The person organising that study is responsible for explaining the project to you before you give consent.
2. Please ask the researcher any questions you may have about this project, before you decide whether you wish to participate.
3. If you decide, now or at any other stage, that you do not wish your child to participate in the research project, that is entirely your right, and if your child is a patient it will not in any way prejudice any present or future treatment.
4. You will be given an information sheet which describes the research (version 2 dated June 2011). This information sheet is for you to keep and refer to, please read it carefully.
5. If you have any complaints about the way in which this research project has been or is being conducted, please, in the first instance, discuss them with the researcher. If the problems are not resolved, or you wish to comment in any other way, please contact PALS via the hospital switchboard.
6. I / We give permission for my child's GP to be informed of their participation.
7. *I / We give permission for my child's blood samples to be used in ethically approved research which may increase knowledge of SLE and associated conditions.*

**CONSENT**

I/We \_\_\_\_\_ / \_\_\_\_\_

being the parent(s)/guardian(s) of \_\_\_\_\_ agree that the research project has been explained to my/our satisfaction and I/We give permission for our child to take part in this study. I/We have read the notes and the information sheets and understand what the research study involves.

SIGNED Parent (s)/Guardian (s)      PRINTED      DATE

-----

SIGNED (Researcher)      PRINTED      DATE

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Copies: Researcher / Notes / family

**Consent Form for PARTICIPANTS in Research Studies**

Cardio-Vascular risk in children and young people with  
Systemic Lupus Erythematosus (SLE / Lupus)

**NOTES FOR PARTICIPANTS**

1. You have been asked to take part in some research. The person organising that study must explain the project to you before you agree to take part.
2. Please ask the researcher any questions you like about this project, before you decide whether to join the study.
3. If you decide, now or at any other time, that you do not wish to be involved in the research project, just tell us and we will stop the research. If you are a patient your treatment will carry on as normal.
4. You will be given an information sheet which describes the research (version 2 dated June 2011). This information is for you to keep and refer to at any time, please read it carefully.
5. If you have any complaints about the research project, discuss them with the researcher. If the problems are not resolved, or you wish to comment in any other way, please contact PALS via the hospital switchboard.
6. I give permission for my GP to be informed of my participation in this project.
7. *I give permission for my blood samples to be used in ethically approved research which may increase knowledge of SLE and associated conditions.*

**CONSENT**

I \_\_\_\_\_ agree that the research project has been explained to my satisfaction and I agree to take part in this study. I have read the notes and the information sheets about the project, and understand what the research study involves.

SIGNED

PRINTED

DATE

-----

SIGNED (Researcher)

PRINTED

DATE

-----

Copies: Researcher / Notes / family

**Consent Form for PARTICIPANTS in Research Studies**

Cardio-Vascular risk in children and young people with  
Systemic Lupus Erythematosus (SLE / Lupus)

**NOTES FOR PARTICIPANTS**

1. You have been asked to take part in some research. The person organising that study must explain the project to you before you agree to take part.
2. Please ask the researcher any questions you like about this project, before you decide whether to join the study.
3. If you decide, now or at any other time, that you do not wish to be involved in the research project, just tell us and we will stop the research. If you are a patient your treatment will carry on as normal.
4. You will be given an information sheet which describes the research (version 2 dated June 2011). This information is for you to keep and refer to at any time, please read it carefully.
5. If you have any complaints about the research project, discuss them with the researcher. If the problems are not resolved, or you wish to comment in any other way, please contact PALS via the hospital switchboard.
6. I give permission for my GP to be informed of my participation in this project.
7. *I give permission for my blood samples to be used in ethically approved research which may increase knowledge of SLE and associated conditions.*

**CONSENT**

I \_\_\_\_\_ agree that the research project has been explained to my satisfaction and I agree to take part in this study. I have read the notes and the information sheets about the project, and understand what the research study involves.

SIGNED

PRINTED

DATE

-----

SIGNED (Researcher)

PRINTED

DATE

-----

Copies: Researcher / Notes / family

Date

Dr xxxx

Dear Doctor

I am writing to inform you that xxxx has been enrolled into the Cardio-Vascular risk in children and young people with Systemic Lupus Erythematosus (SLE), please find enclosed the information sheet

If you have any questions please don't hesitate to contact me – [TulluK@gosh.nhs.uk](mailto:TulluK@gosh.nhs.uk)

Yours sincerely,

Dr Kjell Tullus PhD FRPCH  
Consultant Paediatric Nephrologist

c.c. family

Date

Dr xxxx

Dear Doctor

I am writing to inform you that xxxx has been enrolled into the Cardio-Vascular risk in children and young people with Systemic Lupus Erythematosus (SLE) study please find enclosed the information sheet

If you have any questions please don't hesitate to contact me – [PilkiC@gosh.nhs.uk](mailto:PilkiC@gosh.nhs.uk)

Yours sincerely,

Dr Clarissa Pilkington MBBS MRCP (paeds)  
Consultant Paediatric Rheumatologist

c.c. family

**The Children's University Hospital, Temple Street, Dublin 1**

**And**

**Great Ormond Street Hospital for Children NHS Foundation Trust**

**Consent Form for PARENTS / GUARDIANS of Children Participating  
in Research Studies**

Cardio-Vascular risk in Children and Young People

**NOTES FOR PARENTS OR GUARDIANS**

1. Your child has been asked to take part in a research study. The person organising that study is responsible for explaining the project to you before you give consent.
2. Please ask the researcher any questions you may have about this project, before you decide whether you wish to participate.
3. If you decide, now or at any other stage, that you do not wish your child to participate in the research project, that is entirely your right, and if your child is a patient it will not in any way prejudice any present or future treatment.
4. You will be given an information sheet which describes the research (version 1 dated March 2012). This information sheet is for you to keep and refer to, please read it carefully.
5. If you have any complaints about the way in which this research project has been or is being conducted, please, in the first instance, discuss them with the researcher.
6. I give permission for my child's GP to be informed of their participation.
7. I give permission for my child's blood samples to be used in ethically approved research which may increase knowledge of cardiovascular risk in children and young people.

**CONSENT**

I/We \_\_\_\_\_ / \_\_\_\_\_

being the parent(s)/guardian(s) of \_\_\_\_\_ agree that the research project has been explained to my/our satisfaction and I/We give permission for our child to take part in this study. I/We have read the notes and the information sheets and understand what the research study involves.

SIGNED Parent (s)/Guardian (s)      PRINTED      DATE

-----

SIGNED (Researcher)      PRINTED      DATE

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## Appendix 4: CUH information sheets and consents







Great Ormond Street  
Hospital for Children  
NHS Trust



## **Research Study Investigating Cardiovascular Risk in Children**

Are you doing bloods on a child aged 10 or older without chronic medical problems?

If so, please consider discussing this study with them.

It involves:

- Taking an extra 7.5ml sample of blood and sending it to biochemistry with the enclosed form

If you would like any further information please call Catherine Quinlan on 087 2032431. I can explain the study to parents and obtain consent.

If it is very late at night the information sheets and consent forms are in the attached envelope.

Thank You



Great Ormond Street  
Hospital for Children  
NHS Trust



## **Research Study Investigating Cardiovascular Risk in Children**

Are you interested in participating in a research study?

Are you 10 years or older?

Would you like to find out more?

This study involves having an extra sample of blood taken, it does not involve any extra needles and will not take any extra time.

If yes please add your name to the research sign-up sheet or contact the Dr Quinlan (main researcher) on 087 2032431.



Great Ormond Street **NHS**  
Hospital for Children  
NHS Trust

## **Information for parents and guardians of young people who are being investigated as CONTROL SUBJECTS in a study investigating cardiovascular risk in children and young people**

We would like to ask for you and your child to help us with this study on the optimal management of the heart and blood vessel changes in patients with SLE. Your child could be investigated as a control subject so we can look at the differences between young people with SLE and young people without SLE.

### **Why is the study being done?**

We know that young adults with SLE have an increased risk of heart attack and stroke compared to their unaffected peers. We are trying to identify those at most risk earlier in life to help minimise the long term problems.

We want to do that by gathering information about how blood vessels are affected by SLE and what causes those changes. To ensure accurate results we need to investigate a control group of young people without SLE.

### **How is the study to be done?**

We will collect a urine sample and 7.5mls (1½ teaspoons) of blood, which will be analysed for known indicators of blood vessel damage.

We will ask your permission to use some of the blood taken for ethically approved research such as tests for analysing cardio-vascular health in children without SLE. These tests will be analysed in:

- The Children's University Hospital, Temple Street, Dublin 1
- Our Lady's Children's Hospital, Crumlin, Dublin 12
- Great Ormond Street Hospital for Children NHS Foundation Trust, London

The extra blood samples will be taken with your child's routine blood tests and will involve no extra needles.

If possible, we would like to measure your child's height, weight and blood pressure.

### **Are there risks and discomforts?**

There is of course a small risk that we will discover something abnormal during the research investigation that was previously unknown about your child; we will of course discuss these findings with you, your GP and your child's consultant.

We will use an electronic blood pressure machine, this can be uncomfortable for the duration of the test (approx 30-60 seconds).

### **What are the potential benefits?**

There are no expected benefits for your child but obviously there is a chance we may discover something that needs further investigation and we will refer your child to the appropriate medical team if this occurs.

### **Do we have to take part in this study?**

If you decide that you do not wish your child to participate in this research project, which is entirely your right, it will not in any way prejudice any present or future treatment. If they do enter the study and then wish to withdraw, at a later date, this still applies but we will keep all anonymised data collected until that time.

### **Who can we ask about this study?**

You can ask your family, the doctors you usually see in clinic; you can also discuss anything you may need further information about with Dr Catherine Quinlan who is the main researcher for the Irish site. She can be contacted via the switchboard in Temple Street on 01 878 4200.

### **Who will have access to my child's case notes and research records?**

The information gathered will be stored in a secure data base using their hospital registration number; accessible only to your usual doctors, nurses, members of the research team and representatives of the Research Ethics Committee.

### **What are the arrangements for compensation, should any harm come?**

An independent research ethics committee who believe that it is of minimal risk to your child has approved this project. However, research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm should occur as a result of taking part in this study. No special compensation arrangements have been made for this project but you have the right to claim damages in a court of law. This would require you to prove fault on the part of the hospital or any manufacturer involved.

### **Who do we speak to if problems arise?**

If you have any complaints about the way in which this research project has been, or is being conducted, please discuss them with the researchers. If the problems are not resolved, or you wish to comment in any other way, please contact – PALS via the hospital switchboard 0207 405 9200

### **Information for your GP**

We will write to your GP if we discover something abnormal during the course of the study, to inform them of your child's participation in this study and any relevant results that need further investigation.

## Appendix 5: Data collection proforma



Date

Study Code

# **Cardiovascular Morbidity in Children and Young Adults with Systemic Lupus Erythematosus**

Patient Sticker





Weight	Kg	
Height	Cm	
Blood Pressure	Manual <input type="checkbox"/>	Automated <input type="checkbox"/>
Systolic	mmHg	
Diastolic	mmHg	
Birthweight	Kg	
Gestation	/40	

Last Menstrual Period	Day / Month	
Use of Oral Contraceptive Pill	No <input type="checkbox"/>	Yes <input type="checkbox"/>
If yes, when started?	Month / Year	
Brand name		
Cigarette use	Never <input type="checkbox"/>	Occasional <input type="checkbox"/> Yes <input type="checkbox"/>
If yes, how many	Cigarettes/day	
If yes, when started	Month / Year	
Is there a family Hx of cardiovascular disease?	No <input type="checkbox"/>	Yes <input type="checkbox"/>
If yes, please give details		
What relative(s)?		
At what age?		
CVA/TIA/MI/Angina/other		

**Current Medications**

Name	Dose	Frequency	Name	Dose	Frequency
Prednisolone			Cyclophosphamide		
MMF			Hydroxychloroquin		
Rituximab			Cyclosporin		
Azathioprine			NSAIDs		
Cumulative Prednisolone dose over last 6 months				g	

**Previous Medications**

Cyclophosphamide	
Rituximab	
MMF	
Azathioprine	

Cigarette use	Never <input type="checkbox"/>	Occasional <input type="checkbox"/>	Yes <input type="checkbox"/>
If yes, how many	Cigarettes/day		
If yes, when started	Month / Year		

<b>Physical Activity</b>	
Vigorous Activity	Days per week
Time on one day	Hours / Minutes
Moderate Activity	Days per week
Time on one day	Hours / Minutes
Walking >10 minutes	Days per week
Time on one day	Hours / Minutes / Unsure
Sitting time per day	Hours / Minutes / Unsure

<b>Sun Exposure</b>						
Time outside in Summer on weekdays – 1000 – 1600	Hours/Day					
Time outside in Summer on weekend days – 1000 – 1600	Hours/Day					
Number of red/painful sunburns in last 12 months	1	2	3	4	5	
Outside on a warm sunny day	Never	Rarely	Sometimes	Often	Always	
How often do you wear sunscreen?						
How often do you wear a shirt with sleeves that covers shoulders?						
How often do you wear a hat?						
How often do you stay in the shade/use umbrella?						
How often do you wear sunglasses?						
How often do you spend time in the sun to get a tan?						
Colour of untanned skin	Very Fair	Fair	Olive	Light Brown	Dark Brown	Very Dark



Carotid to SS notch: cm

SS notch to Femoral:

Direct: cm

L Carotid -> R Femoral

	BP	TT(ms) Car-Rad	SD (ms) ECG-Car	SD(ms) ECG-Fem	HR (bpm) Car Rad		PWV (ms)	+/- SD
1								
2								
3								

Indirect: cm

L Carotid -> R Femoral

	BP	TT(ms) Car-Rad	SD (ms) ECG-Car	SD(ms) ECG-Fem	HR (bpm) Car Rad		PWV (ms)	+/- SD
1								
2								
3								

## Appendix 6: CDC BMI charts

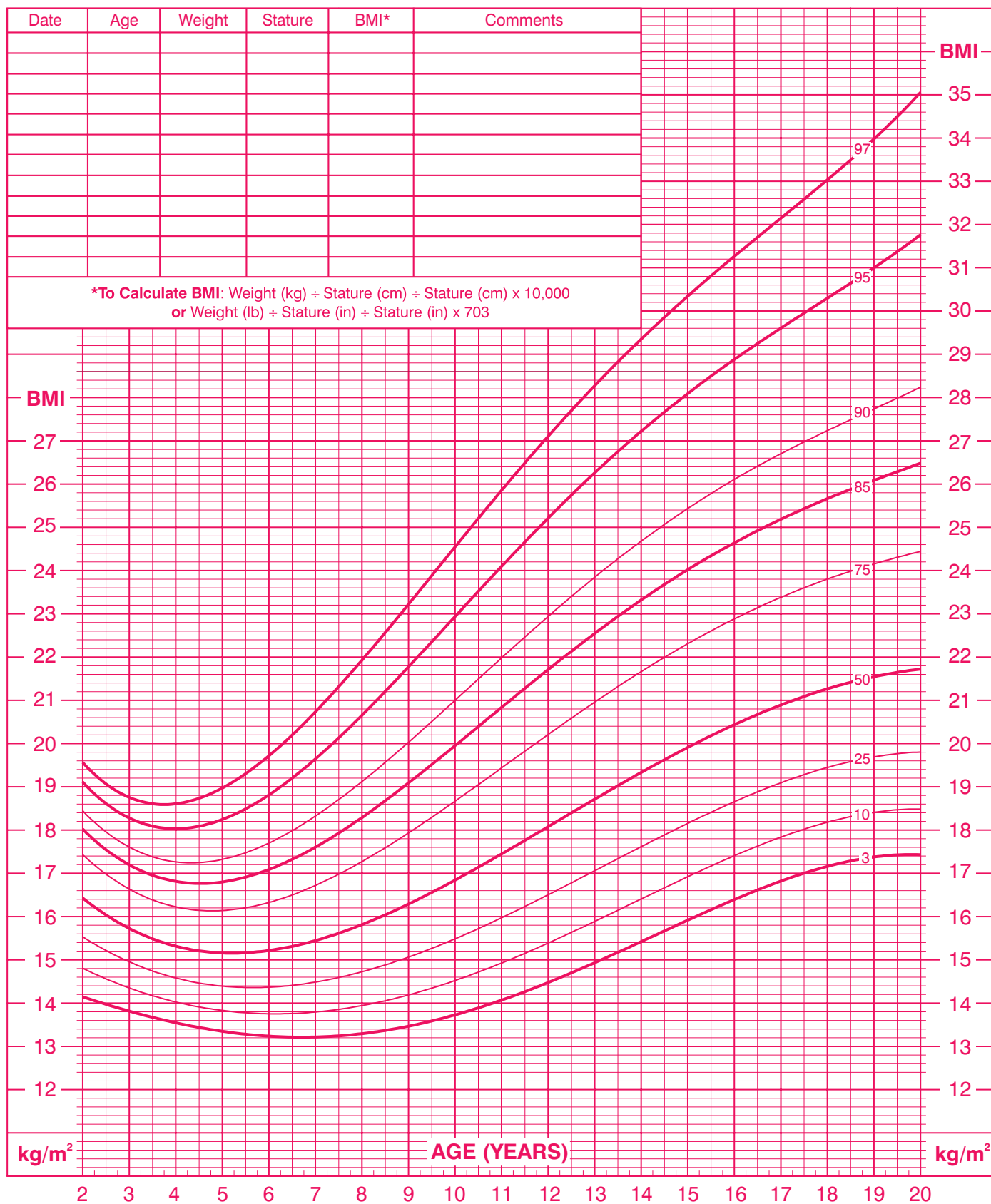


## 2 to 20 years: Girls

### Body mass index-for-age percentiles

NAME \_\_\_\_\_

RECORD # \_\_\_\_\_





## Body mass index-for-age percentiles

NAME \_\_\_\_\_

RECORD # \_\_\_\_\_

[illegible]

## Appendix 7: iPAQ data collection





# **Guidelines for Data Processing and Analysis of the International Physical Activity Questionnaire (IPAQ)**

## **– Short and Long Forms**

**November 2005**

### **Contents**

- 1. Introduction**
  - 2. Uses of IPAQ Instruments**
  - 3. Summary Characteristics of Short and Long Forms**
  - 4. Overview of Continuous and Categorical Analyses of IPAQ**
  - 5. Protocol for Short Form**
  - 6. Protocol for Long Form**
  - 7. Data Processing Rules**
  - 8. Summary Algorithms**
- 
- |                    |                                                        |
|--------------------|--------------------------------------------------------|
| <b>Appendix 1.</b> | <b>At A Glance IPAQ Scoring Protocol – Short Forms</b> |
| <b>Appendix 2.</b> | <b>At A Glance IPAQ Scoring Protocol – Long Forms</b>  |

## 1. Introduction

This document describes recommended methods of scoring the data derived from the telephone / interview administered and self-administered IPAQ short and long form instruments. The methods outlined provide a revision to earlier scoring protocols for the IPAQ short form and provide for the first time a comparable scoring method for IPAQ long form. Latest versions of IPAQ instruments are available from [www.ipaq.ki.se](http://www.ipaq.ki.se).

Although there are many different ways to analyse physical activity data, to date there is no formal consensus on a 'correct' method for defining or describing levels of physical activity based on self-report population surveys. The use of different scoring protocols makes it very difficult to compare within and between countries, even when the same instrument has been used. Use of these scoring methods will enhance the comparability between surveys, provided identical sampling and survey methods have been used.

## 2. Uses of IPAQ Instruments

IPAQ short form is an instrument designed primarily for population surveillance of physical activity among adults. It has been developed and tested for use in adults (age range of 15-69 years) and until further development and testing is undertaken the use of IPAQ with older and younger age groups is not recommended.

IPAQ short and long forms are sometimes being used as an evaluation tool in intervention studies, but this was not the intended purpose of IPAQ. Users should carefully note the range of domains and types of activities included in IPAQ before using it in this context. Use as an outcome measure in small scale intervention studies is not recommended.

## 3. Summary Characteristics of IPAQ Short and Long Forms

1. IPAQ assesses physical activity undertaken across a comprehensive set of domains including:
  - a. leisure time physical activity
  - b. domestic and gardening (yard) activities
  - c. work-related physical activity
  - d. transport-related physical activity;
2. The IPAQ **short** form asks about three specific types of activity undertaken in the four domains introduced above. The specific types of activity that are assessed are walking, moderate-intensity activities and vigorous-intensity activities.
3. The items in the **short** IPAQ form were structured to provide separate scores on walking, moderate-intensity and vigorous-intensity activity. Computation of the total score for the short form requires summation of the duration (in minutes) and frequency (days) of walking, moderate-intensity and vigorous-intensity activities. Domain specific estimates cannot be estimated.

4. The IPAQ **long** form asks details about the specific types of activities undertaken within each of the four domains. Examples include walking for transportation and moderate-intensity leisure-time activity.
5. The items in the **long** IPAQ form were structured to provide separate domain specific scores for walking, moderate-intensity and vigorous-intensity activity within each of the work, transportation, domestic chores and gardening (yard) and leisure-time domains. Computation of the total scores for the long form requires summation of the duration (in minutes) and frequency (days) for all the types of activities in all domains. Domain specific scores or activity specific sub-scores may be calculated. Domain specific scores require summation of the scores for walking, moderate-intensity and vigorous-intensity activities within the specific domain, whereas activity-specific scores require summation of the scores for the specific type of activity across domains.

#### **4. Overview of Continuous and Categorical Analyses of IPAQ**

Both categorical and continuous indicators of physical activity are possible from both IPAQ forms. However, given the non-normal distribution of energy expenditure in many populations, it is suggested that the continuous indicator be presented as median minutes/week or median MET–minutes/week rather than means (such as mean minutes/week or mean MET-minutes/week).

##### **4.1 Continuous Variables**

Data collected with IPAQ can be reported as a continuous measure. One measure of the volume of activity can be computed by weighting each type of activity by its energy requirements defined in METs to yield a score in MET–minutes. METs are multiples of the resting metabolic rate and a MET-minute is computed by multiplying the MET score of an activity by the minutes performed. MET-minute scores are equivalent to kilocalories for a 60 kilogram person. Kilocalories may be computed from MET-minutes using the following equation:  $\text{MET-min} \times (\text{weight in kilograms}/60 \text{ kilograms})$ . MET-minutes/day or MET-minutes/week can be presented although the latter is more frequently used and is thus suggested.

Details for the computation for summary variables from IPAQ short and long forms are detailed below. As there are no established thresholds for presenting MET-minutes, the IPAQ Research Committee propose that these data are reported as comparisons of median values and interquartile ranges for different populations.

##### **4.2 Categorical Variable: Rationale for Cut Point Values**

There are three levels of physical activity proposed to classify populations:

1. Low
2. Moderate
3. High

The algorithms for the short and long forms are defined in more detail in Sections 5.3 and 6.3, respectively. Rules for data cleaning and processing prior to computing the algorithms appear in Section 7.

Regular participation is a key concept included in current public health guidelines for physical activity.<sup>1</sup> Therefore, both the total volume and the number of days/sessions are included in the IPAQ analysis algorithms.

The criteria for these levels have been set taking into account that IPAQ asks questions in all domains of daily life, resulting in higher median MET-minutes estimates than would have been estimated from leisure-time participation alone. The criteria for these three levels are shown below.

Given that measures such as IPAQ assess total physical activity in all domains, the “leisure time physical activity” based public health recommendation of 30 minutes on most days will be achieved by most adults in a population. Although widely accepted as a goal, in absolute terms 30 minutes of moderate-intensity activity is low and broadly equivalent to the background or basal levels of activity adult individuals would accumulate in a day. Therefore a new, higher cutpoint is needed to describe the levels of physical activity associated with health benefits for measures such as IPAQ, which report on a broad range of domains of physical activity.

### **‘High’**

This category was developed to describe higher levels of participation. Although it is known that greater health benefits are associated with increased levels of activity there is no consensus on the exact amount of activity for maximal benefit. In the absence of any established criteria, the IPAQ Research Committee proposes a measure which equates to approximately at least one hour per day or more, of at least moderate-intensity activity above the basal level of physical activity. Considering that basal activity may be considered to be equivalent to approximately 5000 steps per day, it is proposed that “high active” category be considered as those who move at least 12,500 steps per day, or the equivalent in moderate and vigorous activities. This represents at least an hour more moderate-intensity activity over and above the basal level of activity, or half an hour of vigorous-intensity activity over and above basal levels daily. These calculations were based on emerging results of pedometers studies.<sup>2</sup>

This category provides a higher threshold of measures of total physical activity and is a useful mechanism to distinguish variation in population groups. Also it could be used to set population targets for health-enhancing physical activity when multi-domain instruments, such as IPAQ are used.

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<sup>1</sup> Pate RR, Pratt M, Blair SN, Haskell WL, Macera CA, Bouchard C et al. Physical activity and public health. A recommendation from the Centers for Disease Control and Prevention and the American College of Sports Medicine. *Journal of American Medical Association* 1995; 273(5):402-7. and U.S. Department of Health and Human Services. *Physical Activity and Health: A Report of the Surgeon General*. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, The Presidents' Council on Physical Fitness and Sports: Atlanta, GA:USA. 1996.

<sup>2</sup> Tudor-Locke C, Bassett DR Jr. How many steps/day are enough? Preliminary pedometer indices for public health. *Sports Med.* 2004;34(1):1-8.

### **'Moderate'**

This category is defined as doing some activity, more than the low active category. It is proposed that it is a level of activity equivalent to “half an hour of at least moderate-intensity PA on most days”, the former leisure time-based physical activity population health recommendation.

### **'Low'**

This category is simply defined as not meeting any of the criteria for either of the previous categories.

## **5. Protocol for IPAQ Short Form**

### **5.1 Continuous Scores**

Median values and interquartile ranges can be computed for walking (W), moderate-intensity activities (M), vigorous-intensity activities (V) and a combined total physical activity score. All continuous scores are expressed in MET-minutes/week as defined below.

### **5.2 MET Values and Formula for Computation of MET-minutes/week**

The selected MET values were derived from work undertaken during the IPAQ Reliability Study undertaken in 2000-2001<sup>3</sup>. Using the Ainsworth et al. Compendium (*Med Sci Sports Med* 2000) an average MET score was derived for each type of activity. For example; all types of walking were included and an average MET value for walking was created. The same procedure was undertaken for moderate-intensity activities and vigorous-intensity activities. The following values continue to be used for the analysis of IPAQ data: Walking = 3.3 METs, Moderate PA = 4.0 METs and Vigorous PA = 8.0 METs. Using these values, four continuous scores are defined:

Walking MET-minutes/week = 3.3 \* walking minutes \* walking days

Moderate MET-minutes/week = 4.0 \* moderate-intensity activity minutes \* moderate days

Vigorous MET-minutes/week = 8.0 \* vigorous-intensity activity minutes \* vigorous-intensity days

Total physical activity MET-minutes/week = sum of Walking + Moderate + Vigorous MET-minutes/week scores.

### **5.3 Categorical Score**

#### **Category 1 Low**

This is the lowest level of physical activity. Those individuals who not meet criteria for Categories 2 or 3 are considered to have a 'low' physical activity level.

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<sup>3</sup> Craig CL, Marshall A, Sjostrom M et al. International Physical Activity Questionnaire: 12 country reliability and validity *Med Sci Sports Exerc* 2003;August



## **Category 2 Moderate**

The pattern of activity to be classified as 'moderate' is either of the following criteria:

- a) 3 or more days of vigorous-intensity activity of at least 20 minutes per day  
**OR**
- b) 5 or more days of moderate-intensity activity and/or walking of at least 30 minutes per day  
**OR**
- c) 5 or more days of any combination of walking, moderate-intensity or vigorous intensity activities achieving a minimum Total physical activity of at least 600 MET-minutes/week.

Individuals meeting at least one of the above criteria would be defined as accumulating a minimum level of activity and therefore be classified as 'moderate'. See Section 7.5 for information about combining days across categories.

## **Category 3 High**

A separate category labelled 'high' can be computed to describe higher levels of participation.

The two criteria for classification as 'high' are:

- a) vigorous-intensity activity on at least 3 days achieving a minimum Total physical activity of at least 1500 MET-minutes/week  
**OR**
- b) 7 or more days of any combination of walking, moderate-intensity or vigorous-intensity activities achieving a minimum Total physical activity of at least 3000 MET-minutes/week.

See Section 7.5 for information about combining days across categories.

## **5.4 Sitting Question in IPAQ Short Form**

The IPAQ sitting question is an additional indicator variable of time spent in sedentary activity and is not included as part of any summary score of physical activity. Data on sitting should be reported as median values and interquartile ranges. To-date there are few data on sedentary (sitting) behaviours and no well-accepted thresholds for data presented as categorical levels.

## **6. Protocol for IPAQ Long Form**

The long form of IPAQ asks in detail about walking, moderate-intensity and vigorous-intensity physical activity in each of the four domains. Note: asking more detailed questions regarding physical activity within domains is likely to produce higher prevalence estimates than the more generic IPAQ short form.

## 6.1 Continuous Score

Data collected with the IPAQ long form can be reported as a continuous measure and reported as median MET-minutes. Median values and interquartile ranges can be computed for walking (W), moderate-intensity activities (M), and vigorous-intensity activities (V) within each domain using the formulas below. Total scores may also be calculated for walking (W), moderate-intensity activities (M), and vigorous-intensity activities (V); for each domain (work, transport, domestic and garden, and leisure) and for an overall grand total.

## 6.2 MET Values and Formula for Computation of MET-minutes

### Work Domain

Walking MET-minutes/week at work =  $3.3 * \text{walking minutes} * \text{walking days at work}$

Moderate MET-minutes/week at work =  $4.0 * \text{moderate-intensity activity minutes} * \text{moderate-intensity days at work}$

Vigorous MET-minutes/week at work =  $8.0 * \text{vigorous-intensity activity minutes} * \text{vigorous-intensity days at work}$

Total Work MET-minutes/week = sum of Walking + Moderate + Vigorous MET-minutes/week scores at work.

### Active Transportation Domain

Walking MET-minutes/week for transport =  $3.3 * \text{walking minutes} * \text{walking days for transportation}$

Cycle MET-minutes/week for transport =  $6.0 * \text{cycling minutes} * \text{cycle days for transportation}$

Total Transport MET-minutes/week = sum of Walking + Cycling MET-minutes/week scores for transportation.

### Domestic and Garden [Yard Work] Domain

Vigorous MET-minutes/week yard chores =  $5.5 * \text{vigorous-intensity activity minutes} * \text{vigorous-intensity days doing yard work}$  (**Note:** the MET value of 5.5 indicates that vigorous garden/yard work should be considered a moderate-intensity activity for scoring and computing total moderate intensity activities.)

Moderate MET-minutes/week yard chores =  $4.0 * \text{moderate-intensity activity minutes} * \text{moderate-intensity days doing yard work}$

Moderate MET-minutes/week inside chores =  $3.0 * \text{moderate-intensity activity minutes} * \text{moderate-intensity days doing inside chores}$ .

Total Domestic and Garden MET-minutes/week = sum of Vigorous yard + Moderate yard + Moderate inside chores MET-minutes/week scores.

### Leisure-Time Domain

Walking MET-minutes/week leisure =  $3.3 * \text{walking minutes} * \text{walking days in leisure}$

Moderate MET-minutes/week leisure =  $4.0 * \text{moderate-intensity activity minutes} * \text{moderate-intensity days in leisure}$

Vigorous MET-minutes/week leisure =  $8.0 * \text{vigorous-intensity activity minutes} * \text{vigorous-intensity days in leisure}$

Total Leisure-Time MET-minutes/week = sum of Walking + Moderate + Vigorous MET-minutes/week scores in leisure.

### **Total Scores for all Walking, Moderate and Vigorous Physical Activities**

Total Walking MET-minutes/week = Walking MET-minutes/week (at Work + for Transport + in Leisure)

Total Moderate MET-minutes/week total = Moderate MET-minutes/week (at Work + Yard chores + inside chores + in Leisure time) + Cycling Met-minutes/week for Transport + Vigorous Yard chores MET-minutes/week

Total Vigorous MET-minutes/week = Vigorous MET-minutes/week (at Work + in Leisure)

**Note:** Cycling MET value and Vigorous garden/yard work MET value fall within the coding range of moderate-intensity activities.

### **Total Physical Activity Scores**

An overall total physical activity MET-minutes/week score can be computed as:

Total physical activity MET-minutes/week = sum of Total (Walking + Moderate + Vigorous) MET-minutes/week scores.

This is equivalent to computing:

Total physical activity MET-minutes/week = sum of Total Work + Total Transport + Total Domestic and Garden + Total Leisure-Time MET-minutes/week scores.

As there are no established thresholds for presenting MET-minutes, the IPAQ Research Committee proposes that these data are reported as comparisons of median values and interquartile ranges for different populations.

## **6.3 Categorical Score**

As noted earlier, regular participation is a key concept included in current public health guidelines for physical activity.<sup>4</sup> Therefore, both the total volume and the number of day/sessions are included in the IPAQ analysis algorithms. There are three levels of physical activity proposed to classify populations – 'low', 'moderate', and 'high'. The criteria for these levels are the same as for the IPAQ short [described earlier in Section 4.2]

### **Category 1 Low**

This is the lowest level of physical activity. Those individuals who not meet criteria for Categories 2 or 3 are considered 'low'.

### **Category 2 Moderate**

The pattern of activity to be classified as 'moderate' is either of the following criteria:

- d) 3 or more days of vigorous-intensity activity of at least 20 minutes per day

**OR**

- e) 5 or more days of moderate-intensity activity and/or walking of at least 30 minutes per day

**OR**

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<sup>4</sup> Pate RR, Pratt M, Blair SN, Haskell WL, Macera CA, Bouchard C et al. Physical activity and public health. A recommendation from the Centers for Disease Control and Prevention and the American College of Sports Medicine. *Journal of American Medical Association* 1995; 273(5):402-7. and U.S. Department of Health and Human Services. *Physical Activity and Health: A Report of the Surgeon General*. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, The Presidents' Council on Physical Fitness and Sports: Atlanta, GA:USA. 1996.

- f) 5 or more days of any combination of walking, moderate-intensity or vigorous-intensity activities achieving a minimum Total physical activity of at least 600 MET-minutes/week.

Individuals meeting at least one of the above criteria would be defined as accumulating a moderate level of activity. See Section 7.5 for information about combining days across categories.

### **Category 3 High**

A separate category labelled 'high' can be computed to describe higher levels of participation.

The two criteria for classification as 'high' are:

- a) vigorous-intensity activity on at least 3 days achieving a minimum Total physical activity of at least 1500 MET-minutes/week
- OR**
- b) 7 or more days of any combination of walking, moderate-intensity or vigorous-intensity activities achieving a minimum Total physical activity of at least 3000 MET-minutes/week.

See Section 7.5 for information about combining days across categories.

## **6.4 IPAQ Sitting Question IPAQ Long Form**

The IPAQ sitting question is an additional indicator variable and is not included as part of any summary score of physical activity. To-date there are few data on sedentary (sitting) behaviours and no well-accepted thresholds for data presented as categorical levels. For the sitting question 'Minutes' is used as the indicator to reflect time spent in sitting rather than MET-minutes which would suggest an estimate of energy expenditure.

IPAQ long assesses an estimate of sitting on a typical weekday, weekend day and time spent sitting during travel (see transport domain questions).

### **Summary sitting variables include**

Sitting Total Minutes/week = weekday sitting minutes\* 5 weekdays + weekend day sitting minutes\* 2 weekend days

Average Sitting Total Minutes/day = (weekday sitting minutes\* 5 weekdays + weekend day sitting minutes\* 2 weekend days) / 7

**Note:** The above calculation of 'Sitting Total' excludes time spent sitting during travel because the introduction in IPAQ long directs the responder to NOT include this component as it would have already been captured under the Transport section. If a summary sitting variable including time spent sitting for transport is required, it should be calculated by adding the time reported (travelling in a motor vehicle) under transport to the above formula. Care should be taken in reporting these alternate data to clearly distinguish the 'total sitting' variable from a 'total sitting – including transport' variable.

## **7. Data Processing Rules**

In addition to a standardized approach to computing categorical and continuous measures of physical activity, it is necessary to undertake standard methods for the cleaning and treatment of IPAQ datasets. The use of different approaches and rules would introduce variability and reduce the comparability of data.

There are no established rules for data cleaning and processing on physical activity. Thus, to allow more accurate comparisons across studies IPAQ Research Committee has established and recommends the following guidelines:

### **7.1 Data Cleaning**

- I. Any responses to duration (time) provided in the hours and minutes response option should be converted from hours and minutes into minutes.
- II. To ensure that responses in 'minutes' were not entered in the 'hours' column by mistake during self-completion or during data entry process, values of '15', '30', '45', '60' and '90' in the 'hours' column should be converted to '15', '30', '45', '60' and '90' minutes, respectively, in the minutes column.
- III. In some cases duration (time) will be reported as weekly (not daily) e.g., VWHRS, VWMINS. These data should be converted into an average daily time by dividing by 7.
- IV. If 'don't know' or 'refused' or data are missing for time or days then that case is removed from analysis.

**Note:** Both the number of days *and* daily time are required for the creation of categorical and continuous summary variables

### **7.2 Maximum Values for Excluding Outliers**

This rule is to exclude data which are unreasonably high; these data are to be considered outliers and thus are excluded from analysis. All cases in which the sum total of all Walking, Moderate and Vigorous time variables is greater than 960 minutes (16 hours) should be excluded from the analysis. This assumes that on average an individual of 8 hours per day is spent sleeping.

The 'days' variables can take the range 0-7 days, or 8, 9 (don't know or refused); values greater than 9 should not be allowed and those cases excluded from analysis.

### **7.3 Minimum Values for Duration of Activity**

Only values of 10 or more minutes of activity should be included in the calculation of summary scores. The rationale being that the scientific evidence indicates that episodes or bouts of at least 10 minutes are required to achieve health benefits. Responses of less than 10 minutes [and their associated days] should be re-coded to 'zero'.

## **7.4 Truncation of Data Rules**

This rule attempts to normalize the distribution of levels of activity which are usually skewed in national or large population data sets.

In IPAQ short - it is recommended that all Walking, Moderate and Vigorous time variables exceeding '3 hours' or '180 minutes' are truncated (that is re-coded) to be equal to '180 minutes' in a new variable. This rule permits a maximum of 21 hours of activity in a week to be reported for each category (3 hours \* 7 days).

In IPAQ long – the truncation process is more complicated, but to be consistent with the approach for IPAQ short requires that the variables total Walking, total Moderate-intensity and total Vigorous-intensity activity are calculated and then, for each of these summed behaviours, the total value should be truncated to 3 hours (180 minutes).

When analysing the data as categorical variable or presenting median and interquartile ranges of the MET-minute scores, the application of the truncation rule will not affect the results. This rule does have the important effect of preventing misclassification in the 'high' category. For example, an individual who reports walking for 10 minutes on 6 days and 12 hours of moderate activity on one day could be coded as 'high' because this pattern meets the '7 day' and "3000 MET-min" criteria for 'high'. However, this uncommon pattern of activity is unlikely to yield the health benefits that the 'high' category is intended to represent.

Although using median is recommended due to the skewed distribution of scores, if IPAQ data are analysed and presented as a continuous variable using mean values, the application of the truncation rule will produce slightly lower mean values than would otherwise be obtained.

## **7.5 Calculating MET-minute/week Scores**

Data processing rules 7.2, 7.3, and 7.4 deals first with excluding outlier data, then secondly, with recoding minimum values and then finally dealing with high values. These rules will ensure that highly active people remain classified as 'high', while decreasing the chances that less active individuals are misclassified and coded as 'high'.

Using the resulting variables, convert time and days to MET-minute/week scores [see above Sections 5.2 and 6.2; METS x days x daily time].

## **7.6 Calculating Total Days for Presenting Categorical Data on Moderate and High Levels**

Presenting IPAQ data using categorical variables requires the total number of 'days' on which all physical activity was undertaken to be assessed. This is difficult because frequency in 'days' is asked separately for walking, moderate-intensity and vigorous-intensity activities, thus allowing the total number of 'days' to range from a minimum

of 0 to a maximum of 21 'days' per week in IPAQ short and higher in IPAQ long. The IPAQ instrument does not record if different types of activity are undertaken on the same day.

In calculating 'moderately active', the primary requirement is to identify those individuals who undertake activity on at least '5 days'/week [see Sections 4.2 and 5.3]. Individuals who meet this criterion should be coded in a new variable called "*at least five days*" and this variable should be used to identify those meeting criterion b) at least 30 minutes of moderate-intensity activity and/or walking; and those meeting criterion c) any combination of walking, moderate-intensity or vigorous-intensity activities achieving a minimum of 600 MET-minutes/week.

Below are two examples showing this coding in practice:

- i) an individual who reports '2 days of moderate-intensity' and '3 days of walking' should be coded as a value indicating "*at least five days*";
- ii) an individual reporting '2 days of vigorous-intensity', '2 days of moderate-intensity' and '2 days of walking' should be coded as a value to indicate "*at least five days*" [even though the actual total is 6].

The original frequency of 'days' for each type of activity should remain in the data file for use in the other calculations.

The same approach as described above is used to calculate total days for computing the 'high' category. The primary requirement according to the stated criteria is to identify those individuals who undertake a combination of walking, moderate-intensity and or vigorous-intensity activity on at least 7 days/week [See section 4.2]. Individuals who meet this criterion should be coded as a value in a new variable to reflect "*at least 7 days*".

Below are two examples showing this coding in practice:

- i) an individual who reports '4 days of moderate-intensity' and '3 days of walking' should be coded as the new variable "*at least 7 days*".
- ii) an individual reporting '3 days of vigorous-intensity', '3 days moderate-intensity' and '3 days walking' should be coded as "*at least 7 days*" [even though the total adds to 9] .

## **8. Summary algorithms**

The algorithms in Appendix 1 and Appendix 2 to this document show how these rules work in an analysis plan, to develop the categories 1 [Low], 2 [Moderate], and 3 [High] levels of activity.

**IPAQ Research Committee  
November 2005**

# APPENDIX 1

## At A Glance IPAQ Scoring Protocol (Short Forms)

### Continuous Score

Expressed as MET-min per week: MET level x minutes of activity/day x days per week

#### *Sample Calculation*

#### **MET levels**

Walking = 3.3 METs

Moderate Intensity = 4.0 METs

Vigorous Intensity = 8.0 METs

#### **MET-minutes/week for 30 min/day, 5 days**

$3.3 \times 30 \times 5 = 495$  MET-minutes/week

$4.0 \times 30 \times 5 = 600$  MET-minutes/week

$8.0 \times 30 \times 5 = 1,200$  MET-minutes/week

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**TOTAL = 2,295 MET-minutes/week**

Total MET-minutes/week = Walk (METs\*min\*days) + Mod (METs\*min\*days) + Vig (METs\*min\*days)

### Categorical Score- three levels of physical activity are proposed

#### 1. **Low**

- No activity is reported **OR**
- Some activity is reported but not enough to meet Categories 2 or 3.

#### 2. **Moderate**

Either of the following 3 criteria

- 3 or more days of vigorous activity of at least 20 minutes per day **OR**
- 5 or more days of moderate-intensity activity and/or walking of at least 30 minutes per day **OR**
- 5 or more days of any combination of walking, moderate-intensity or vigorous-intensity activities achieving a minimum of at least 600 MET-minutes/week.

#### 3. **High**

Any one of the following 2 criteria

- Vigorous-intensity activity on at least 3 days and accumulating at least 1500 MET-minutes/week **OR**
- 7 or more days of any combination of walking, moderate- or vigorous-intensity activities accumulating at least 3000 MET-minutes/week

Please review the full document “Guidelines for the data processing and analysis of the International Physical Activity Questionnaire” for more detailed description of IPAQ analysis and recommendations for data cleaning and processing [[www.ipaq.ki.se](http://www.ipaq.ki.se)].



## APPENDIX 2

### At A Glance IPAQ Scoring Protocol (Long Forms)

#### Continuous Score

Expressed as MET-minutes per week: MET level x minutes of activity/day x days per week

#### *Sample Calculation*

##### **MET levels**

Walking at work= 3.3 METs

Cycling for transportation= 6.0 METs

Moderate yard work= 4.0 METs

Vigorous intensity in leisure= 8.0 METs

##### **MET-minutes/week for 30 min/day, 5 days**

$3.3 \times 30 \times 5 = 495$  MET-minutes/week

$6.0 \times 30 \times 5 = 900$  MET-minutes/week

$4.0 \times 30 \times 5 = 600$  MET-minutes/week

$8.0 \times 30 \times 5 = 1,200$  MET-minutes/week

---

**TOTAL = 3,195 MET-minutes/week**

#### Domain Sub Scores

Total MET-minutes/week at **work** = Walk (METs\*min\*days) + Mod (METs\*min\*days) + Vig (METs\*min\*days) at work

Total MET-minutes/week for **transportation** = Walk (METs\*min\*days) + Cycle (METs\*min\*days) for transportation

Total MET-minutes/week from **domestic and garden** = Vig (METs\*min\*days) yard work + Mod (METs\*min\*days) yard work + Mod (METs\*min\*days) inside chores

Total MET-minutes/week in **leisure-time** = Walk (METs\*min\*days) + Mod (METs\*min\*days) + Vig (METs\*min\*days) in leisure-time

#### Walking, Moderate-Intensity and Vigorous-Intensity Sub Scores

Total **Walking** MET-minutes/week = Walk MET-minutes/week (at Work + for Transport + in Leisure)

Total **Moderate** MET-minutes/week = Cycle MET-minutes/week for Transport + Mod MET-minutes/week (Work + Yard chores + Inside chores + Leisure) + Vigorous Yard chores MET-minutes

**Note:** The above is a total moderate activities only score. If you require a total of all moderate-intensity physical activities you would sum Total Walking and Total Moderate

Total **Vigorous** MET-minutes/week = Vig MET-minutes/week (at Work + in Leisure)

#### Total Physical Activity Score

**Total Physical Activity** MET-minutes/week = **Walking** MET-minutes/week + **Moderate** MET-minutes/week + Total **Vigorous** MET-minutes/week

Continued ..

## **Also**

**Total** Physical Activity MET-minutes/week = Total MET-minutes/week (at Work + for Transport + in Chores + in Leisure)

## **Categorical Score- three levels of physical activity are proposed**

### **1. Low**

No activity is reported **OR**

- a. Some activity is reported but not enough to meet Categories 2 or 3.

### **2. Moderate**

Either of the following 3 criteria

- a. 3 or more days of vigorous-intensity activity of at least 20 minutes per day **OR**
- b. 5 or more days of moderate-intensity activity and/or walking of at least 30 minutes per day **OR**
- c. 5 or more days of any combination of walking, moderate-intensity or vigorous-intensity activities achieving a minimum of at least 600 MET-min/week.

### **3. High**

Any one of the following 2 criteria

- Vigorous-intensity activity on at least 3 days and accumulating at least 1500 MET-minutes/week **OR**
- 7 or more days of any combination of walking, moderate- or vigorous- intensity activities accumulating at least 3000 MET-minutes/week

**Please review the full document “Guidelines for the data processing and analysis of the International Physical Activity Questionnaire” for more detailed description of IPAQ analysis and recommendations for data cleaning and processing [[www.ipaq.ki.se](http://www.ipaq.ki.se)].**

## Appendix 8: Adipoikine ELISA product information sheet



# Quantikine<sup>®</sup> ELISA

## Human Leptin Immunoassay

Catalog Number DLP00

SLP00

PDLP00

For the quantitative determination of human Leptin concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.  
For research use only. Not for use in diagnostic procedures.

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## INTRODUCTION

Human Leptin (gene name OB) is a 16 kDa, 146 amino acid (aa) residue, non-glycosylated polypeptide that regulates adipose tissue mass and energy balance (1-6). The name Leptin is derived from the Greek (leptos, or "thin") because of its ability to reduce fat stores (7). In mice (ob/ob) and humans, inactivating mutations of the OB gene can cause obesity (1-6). Mature human Leptin shares 87% and 84% aa identity with mouse and rat Leptin, respectively (1, 8). Human Leptin is active in both the mouse and rat systems (9, 10). Leptin is expressed almost exclusively by adipocytes and its production is influenced by hormones, cytokines and nutrients (5, 8, 11). For example, Leptin expression is enhanced by insulin and glucocorticoids, which are associated with positive energy balance, while catecholamines decrease Leptin production during negative energy balance (5). It circulates in the plasma, crosses the blood-brain barrier, and is present in human breast milk (3-6, 12).

The human Leptin receptor (designated ObR or LEPR) is a 150 kDa, 1144 aa residue, type I transmembrane glycoprotein of the IL-6 receptor family of Class I cytokine receptors (13, 14). The gene for ObR undergoes considerable splicing, forming variants a-d with cytoplasmic domains of variable length, plus the potentially soluble form ObRe (14, 15). The long form, ObRb (formerly OB RL), is expressed mainly in the hypothalamic arcuate nucleus and is essential for signal transduction (6, 16, 17). Of the short forms, ObRa is ubiquitous, and ObRa, ObRc, and ObRd are all thought to mediate Leptin binding and endocytosis, but not signal transduction (16). Upon binding of Leptin dimers, ObRb dimers may form signaling tetramers with shorter forms (16). Mutations of ObRb can cause obese phenotypes in both the mouse and rat. The mouse mutation (db/db for diabetes) occurs in the cytoplasmic domain, while the rat mutation (fa/fa for fatty) occurs in the extracellular domain of the receptor (18, 19). In a concentration-dependent manner, Leptin signaling can have diverse effects, causing neurons that express pro-opiomelanocortin (POMC) peptides to reduce food intake, and neurons that express neuropeptide Y and agouti-related protein (NpY and AgRP) to increase food intake (4, 6).

Leptin is fundamentally a "starvation signal" that, when low, prompts increased appetite and decreased energy expenditure (4, 6, 10). Adipocytes increase Leptin expression as cell size increases, which should result in depressed appetite and increased energy expenditure (5). However, obese humans are often resistant to these effects of Leptin (3). Leptin resistance is in part due to saturation of the blood-brain transporter, which is influenced by high circulating triglycerides, and in part due to decreased cellular response to Leptin (6). Rarely, obese humans are genetically Leptin-deficient (3-6). Leptin deficiency also influences the immune system, depressing Th1 responses and causing increased frequency of infections (4). Leptin also regulates puberty, blocking the onset of puberty, or of menses if Leptin deficiency exists due to excessive thinness, such as results from starvation, extreme exercise-induced weight loss, anorexia or cancer-induced cachexia (3, 4).

The Quantikine Human Leptin Immunoassay is a 3.5 hour solid phase ELISA designed to measure soluble human Leptin in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human Leptin and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate recombinant Leptin accurately. Results obtained measuring natural human Leptin showed dose-response curves that were parallel to the standard curves obtained using the recombinant Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human Leptin.

## PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Leptin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Leptin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for Leptin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Leptin bound in the initial step. The color development is stopped and the intensity of the color is measured.

## LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples fall outside the dynamic range of the assay, dilute samples appropriately with Calibrator Diluent and repeat the assay. If cell culture supernate samples require large dilutions, perform an intermediate dilution with culture media and the final dilution with the appropriate Calibrator Diluent.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, however, the possibility of interference cannot be excluded.

## TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.



## MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # DLP00	CATALOG # SLP00	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
<b>Leptin Microplate</b>	890573	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Leptin.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
<b>Leptin Conjugate</b>	890574	1 vial	6 vials	21 mL/vial of mouse monoclonal antibody against Leptin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
<b>Leptin Standard</b>	890575	1 vial	6 vials	10 ng/vial of recombinant human Leptin in a buffered protein base with preservative; lyophilized.	
<b>Assay Diluent RD1-19</b>	895467	1 vial	6 vials	11 mL/vial of a buffered protein base with preservatives.	
<b>Calibrator Diluent RD5P Concentrate</b>	895151	1 vial	6 vials	21 mL/vial of a concentrated buffered protein base with preservatives.	
<b>Wash Buffer Concentrate</b>	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. <i>May turn yellow over time.</i>	
<b>Color Reagent A</b>	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
<b>Color Reagent B</b>	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
<b>Stop Solution</b>	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
<b>Plate Sealers</b>	N/A	4 strips	24 strips	Adhesive strips.	

\* Provided this is within the expiration date of the kit.

DLP00 contains sufficient materials to run an ELISA on one 96 well plate.

SLP00 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDLP00). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

## OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human Leptin Controls (optional; available from R&D Systems).

## PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

## SAMPLE COLLECTION & STORAGE

**The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.**

**Cell Culture Supernates** - Remove particulates by centrifugation and assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Plasma** - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

## SAMPLE PREPARATION

Most serum and plasma samples require a 100-fold dilution. A 100-fold dilution may be achieved by adding 10  $\mu\text{L}$  of sample to 990  $\mu\text{L}$  of Calibrator Diluent RD5P (1X).

If samples fall outside the dynamic range of the assay, a lower or higher dilution may be required.

## REAGENT PREPARATION

**Bring all reagents to room temperature before use.**

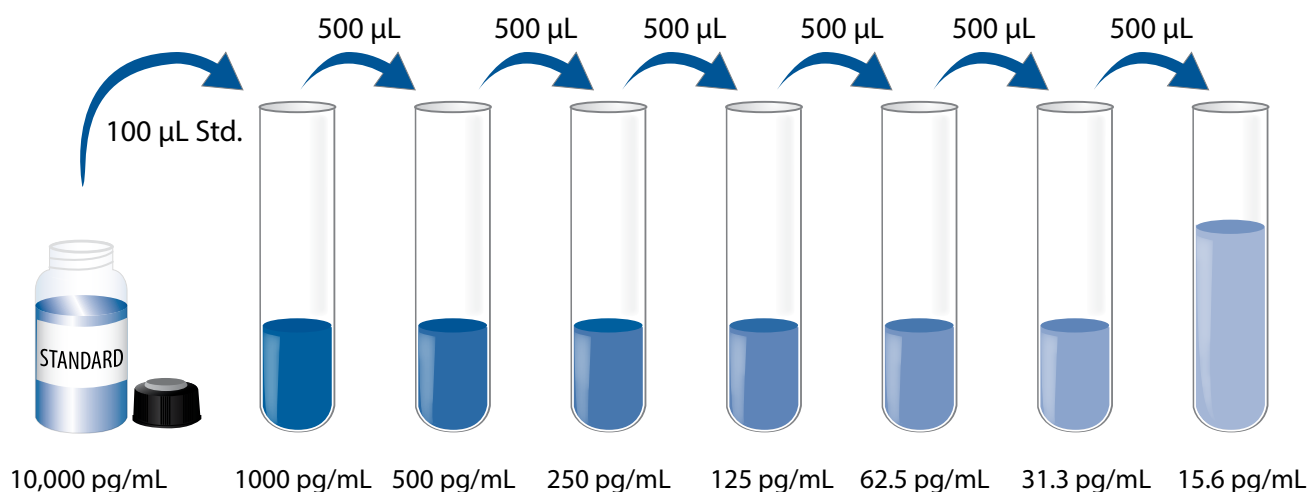
**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

**Substrate Solution** - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200  $\mu$ L of the resultant mixture is required per well.

**Calibrator Diluent RD5P (1X)** - Add 20 mL of Calibrator Diluent RD5P Concentrate to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5P (1X).

**Leptin Standard** - Reconstitute the Leptin Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

**Use polypropylene tubes.** Pipette 900  $\mu$ L of Calibrator Diluent RD5P (1X) into the 1000 pg/mL tube. Pipette 500  $\mu$ L of Calibrator Diluent RD5P (1X) into each of the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (1X) serves as the zero standard (0 pg/mL).



## ASSAY PROCEDURE

**Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.**

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100  $\mu\text{L}$  of Assay Diluent RD1-19 to each well.
4. Add 100  $\mu\text{L}$  of Standard, Control, or sample\* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400  $\mu\text{L}$ ) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200  $\mu\text{L}$  of Leptin Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200  $\mu\text{L}$  of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50  $\mu\text{L}$  of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

\*Serum and plasma samples require dilution. See Sample Preparation section.

## CALCULATION OF RESULTS

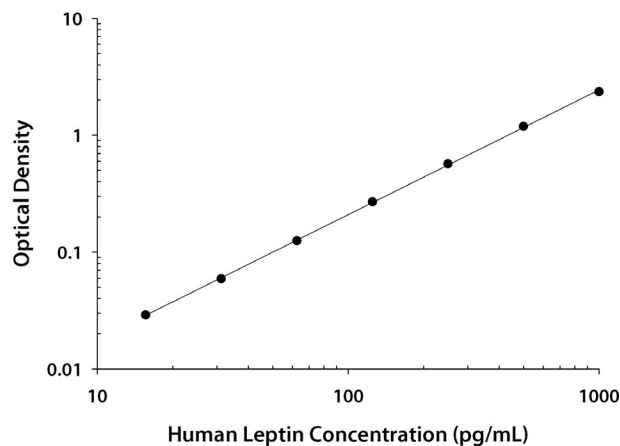
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the Leptin concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

## TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.015 0.015	0.015	—
15.6	0.044 0.044	0.044	0.029
31.3	0.073 0.075	0.074	0.059
62.5	0.136 0.144	0.140	0.125
125	0.282 0.285	0.284	0.269
250	0.581 0.588	0.584	0.569
500	1.195 1.211	1.203	1.188
1000	2.339 2.415	2.377	2.362

## PRECISION

### Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

### Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	64.5	146	621	65.7	146	581
Standard deviation	2.14	4.32	20.0	3.56	6.17	20.6
CV (%)	3.3	3.0	3.2	5.4	4.2	3.5

## RECOVERY

The recovery of Leptin spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=5)	98	94-102%
Serum* (n=5)	95	89-109%
EDTA plasma* (n=5)	99	85-112%
Heparin plasma* (n=5)	90	81-100%
Citrate plasma* (n=5)	95	87-105%

\*Samples were diluted prior to assay as directed in the Sample Preparation section.

## SENSITIVITY

The minimum detectable dose (MDD) of Leptin is typically less than 7.8 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

## LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Leptin were diluted with Calibrator Diluent RD5P (1X) to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=5)	Serum* (n=5)	EDTA plasma* (n=5)	Heparin plasma* (n=5)	Citrate plasma* (n=5)
1:2	Average % of Expected	105	99	99	99	98
	Range (%)	103-107	99-101	97-102	96-104	96-99
1:4	Average % of Expected	109	97	95	97	96
	Range (%)	106-114	94-102	94-99	93-100	93-99
1:8	Average % of Expected	109	92	92	94	93
	Range (%)	107-115	89-95	90-94	90-97	89-97
1:16	Average % of Expected	109	92	91	96	93
	Range (%)	106-113	87-97	86-94	90-100	89-96

\*Samples were diluted prior to assay.

## CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human Leptin produced at R&D Systems.

## SAMPLE VALUES

**Serum** - Samples from apparently healthy volunteers were evaluated for the presence of Leptin in this assay. No medical histories were available for the donors used in this study.

Sample Type	Range (pg/mL)	Mean (pg/mL)
Male Serum (n = 16)	2205-11,149	4760
Female Serum (n = 36)	3877-77,273	20,676

Five additional male serum samples fell below the lowest standard, 15.6 pg/mL, when diluted 100-fold.

**Note:** *Values in EDTA and heparin plasma have been found to be comparable to paired serum samples. Values in citrate plasma have been found to be slightly decreased compared to paired serum, EDTA or heparin plasma samples.*

### Cell Culture Supernates:

Human peripheral blood mononuclear cells ( $5 \times 10^6$  cells/mL) were cultured in RPMI supplemented with 5% fetal calf serum, 50  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10  $\mu$ g/mL PHA. Aliquots of the cell culture supernates were removed on days 1 and 5 and assayed for levels of natural Leptin.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	ND	ND
Stimulated	152	75.9

ND=Non-detectable

BeWo human choriocarcinoma cells ( $1 \times 10^6$  cells/mL) were cultured in F-12 media supplemented with 15% fetal bovine serum. The cells were cultured unstimulated or stimulated with 2  $\mu$ M forskolin and 20  $\mu$ M forskolin. Aliquots of the cell culture supernates were removed on days 1, 2, and 3 and assayed for levels of natural Leptin.

Condition	Day 1 (pg/mL)	Day 2 (pg/mL)	Day 3 (pg/mL)
Unstimulated*	849	1549	1667
2 $\mu$ M forskolin*	1231	1699	2054
20 $\mu$ M forskolin*	1137	1725	2747

\*Samples were diluted 20-fold prior to assay.



## SPECIFICITY

This assay recognizes natural and recombinant human Leptin.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5P (1X) and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range rhLeptin control were assayed for interference. No significant cross-reactivity or interference was observed.

### Recombinant human:

ANG	IL-3
AR	IL-3 sR $\alpha$
CNTF	IL-4
$\beta$ -ECGF	IL-4 sR
EGF	IL-5
Epo	IL-5 sR $\beta$
FGF acidic	IL-6
FGF basic	IL-6 sR
FGF-4	IL-7
FGF-5	IL-8
FGF-6	IL-9
G-CSF	IL-10
GM-CSF	IL-11
sgp130	IL-12
GRO $\alpha$	IL-13
GRO $\beta$	KGF
GRO $\gamma$	LAP (TGF- $\beta$ 1)
HB-EGF	LIF
HGF	M-CSF
IFN- $\gamma$	MCP-1
IGF-I	MIP-1 $\alpha$
IGF-II	MIP-1 $\beta$
IL-1 $\alpha$	$\beta$ -NGF
IL-1 $\beta$	OSM
IL-1ra	PD-ECGF
IL-1 sRI	PDGF-AA
IL-1 sRII	PDGF-AB
IL-2	PDGF-BB
IL-2 sR $\alpha$	PTN

### Recombinant mouse:

GM-CSF
IL-1 $\alpha$
IL-1 $\beta$
IL-3
IL-4
IL-5
IL-5 sR $\alpha$
IL-6
IL-7
IL-9
IL-10
IL-13
Leptin
LIF
MIP-1 $\alpha$
MIP-1 $\beta$
SCF
TNF- $\alpha$

### Recombinant amphibian:

TGF- $\beta$ 5
----------------

### Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF- $\beta$ 1
porcine TGF- $\beta$ 1
porcine TGF- $\beta$ 2

Recombinant human Leptin R/Fc chimera and recombinant mouse Leptin R/Fc chimera do not cross-react in this assay; however, interference was observed at concentrations  $\geq 0.78$  ng/mL.

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**PLATE LAYOUT**

Use this plate layout to record standards and samples assayed.

12								
11								
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## Appendix 9: Subject workflow



# Workflow for Subjects

## 1 week before clinic:

1. Identify suitable patients
  - a. Ask Kjell/Steve/Clarissa/Lucinda
  - b. Look at patient list on PIMS and go through letters on the system
  - c. Contact Joanna in Vasc Phys and see if there is a room free to scan.
2. Phone numbers stored on PIMS
  - a. Initially I explained that I was a research doctor working with Clarissa/Steve/Kjell and wondered if their child would like to help us with a research study. It will take about an hour of their time.
  - b. If they said yes to this then I explained what I was looking at
    - “There is evidence to suggest that adults with lupus are at an increased risk of heart attacks and strokes. But the average age to get lupus is 51 and we simply don’t know if children with lupus are also at an increased risk. In this study we want to see if they are at an increased risk, hopefully figure out why, and then see what we can do about it.”
  - c. Then I went through the process in detail:
    - Before or after their appointment I will meet them and bring them to the research dept – ie Vasc Phys
    - They will have 2 scans both of which will look at their blood vessels.
    - “The first scan is an ultrasound scan of the blood vessels in their neck. I will use it to measure the thickness of the blood vessel wall. It doesn’t hurt and has no side effects. They will need to lie still on a bed for the scan, it takes about 10-15 mins.”
    - “The second scan is used to measure how elastic or bouncy the blood vessels are. It doesn’t hurt and has no side effects. They will need to lie very still and I will hold a small device that looks like a pen on the pulse in their neck and at the top of their leg. (I found top of leg was much more acceptable than groin!) It takes around 15 minutes. This will be repeated 3 times.”
    - After the 2 scans I will ask a few questions about other risks for heart disease – such as how much exercise they take.
    - Then you are free to go.
    - At the clinic IF they are having bloods done anyway then I will take an extra sample which I will use to look for new markers of heart disease. In practice they all have bloods.
    - They can say yes to all or part of this. And I will explain it in full on the day.
  - d. If they said yes then I would contact Vasc Phys (Joanna) and book the room for the required time. And take contact details so I can email or text them a reminder.

### **The day before clinic:**

1. Reconfirm the room with Joanna
2. Email/Text a reminder to patient. Alternatively phone them, just make sure there's a solid plan so they don't leave/get lost
3. Email consultant to remind them the patient is taking part in clinic
4. Print blood forms for:
  - ANA
  - dsDNA
  - ENA
  - C3
  - C4
  - Cholesterol
  - Triglycerides
  - LDL/VLDL/HDL (lipoproteins)
  - ESR
  - FBC
  - LFTs
  - UAUC (urine sample)
  - (Anti CD19 if clinically indicated)
  - Anti cardiolipin antibodies (AC)
  - CRP
  - hsCRP
  - U+E (C127 on system)
  - PTH
  - TFTs
  - C1q
  - AntiC1q (C1QA)
  - IgG/M/A
  - Ferritin (C283)

(All of this amounts to 4.5ml in a brown tube, 2ml in a red tube, 2.4ml in an orange tube and 7.5ml in a white tube.)
5. Print Study serum request form
6. Give blood forms to relevant consultant
7. Print information sheets and consent forms
8. Print proforma and fill in name, MRN and study number (4 digit and generated from random.org). Put in any info already gleaned from notes/letters/PIMS eg DOB, ethnicity, date of diagnosis...
9. Make sure you have enough aliquot tubes – this is tricky and can require some ingenuity and friends in the labs



### **The morning of the study:**

1. Check the room and the equipment
2. Label a CD for the scan
3. Generate a record on the PIMS laptop
4. If patient is having bloods done in phlebotomy then bring 7.5ml serum tube (available on Eagle ward) and request form to phlebotomy in OPD and ask them to do the bloods and send them with the patient or hold them for you – make sure the plan is clear.
5. If doing bloods yourself then make sure you have all the tubes, labels, needles etc – Eagle ward was where I got supplies generally.

### **The study itself:**

1. Explain the process in detail using the parent and child information sheets. Ask parent to sign the consent form and witness it.
2. Measure cIMT as per ALSPAC protocol.
3. Measure PWV as per ALSPAC protocol.
4. Take blood if not being done in phlebotomy.
5. Bring them back to reception/street/OPD.
6. Collect blood and 5ml of urine from OPD.
7. Send bloods to relevant departments.
8. Leave serum to settle at room temp x 1 hr.
9. Save USS scan to CD
10. Record PWV on proforma
11. Spin and separate serum and urine as per ALSPAC protocols.
12. Separate serum to 0.5ml aliquots and urine to 1ml aliquots put in normal freezer x 24 hrs. Label each with date, urine/serum, and initials and subject number.

### **The day after:**

1. Analyse cIMT as per ALSPAC protocol and record on record sheet.
2. Divide aliquots into plastic bags, labelled with date, subject number, initials and how many are in each bag and put into -80 freezer in CVSLE box..
3. Record what's in the freezer on the inventory – this was an excel sheet managed by Jaimie but I'm not sure who's taking over when he leaves.

### **One week later:**

1. Look up lab results on system and record in spreadsheet

## Appendix 10: Good Clinical Practice certification



# CERTIFICATE OF ATTENDANCE

This is to certify that

— *Cathy Quinlan* —

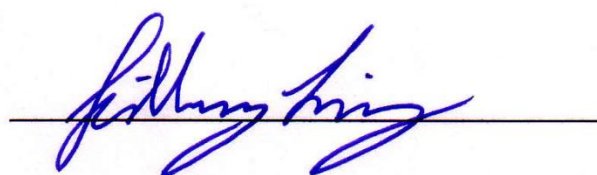
Attended

Overview of Good Clinical Practice

(Full day Session)

UCL Institute of Child Health, London

on 4<sup>th</sup> April 2012



Siobhan Lim

Clinical Research Consultant & Trainer

[www.siobhanlimconsulting.co.uk](http://www.siobhanlimconsulting.co.uk)



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